THE PREVALENCE OF AVIAN MALARIA IN THE MIGRATORY BIRDS OF ARAS-IĞDIR REGION

by

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THE PREVALENCE OF AVIAN MALARIA IN THE MIGRATORY BIRDS OF ARAS-IĞDIR REGION

Avian malaria parasites spread throughout the world and affect a vast range of bird species. *Plasmodium* and *Haemoproteus* that cause avian malaria are popular models to study the ecology and evolution of parasite-host-vector interactions in the world. The studies on avian malaria have focused mostly on the avians host and the malaria parasites. Avian malaria is also a common infection amongst endemic and migratory birds. Malaria infection might cause rapid population declines or species extinctions as a result of the environmental changes and man-made activities. Trying to understand certain aspects of avian malaria such as the extent of parasite diversity, distribution and prevalence in host populations in Aras-Iğdır region, a migratory hotspot in eastern Turkey, comprise the main objectives of this thesis. For these purposes, the prevalence of *Plasmodium* and *Haemoprateus* were confirmed through PCR-based testing. We screened a total of 401 blood samples belonging to 58 bird species of 25 different families. 15 samples were identified as positive and 13 clean chromatograms of a 215 bp cytochrome b fragment of *Plasmodium* and *Haemoprateus* sequences were analyzed together with sequences from the MalAvi database. 13 lineages of *Plasmodium* and *Haemoprateus* from seven bird genera corresponded to nine haplotypes, all of which were previously not recorded. Except one *Plasmodium* sample, all samples clustered into the *Haemoproteus* clade, showing the higher prevelance of the latter in the samples from Aras-Iğdır region. The study results also showed that age-related patterns in avian malaria infections were consistent with the previous studies, where disease survival rates were highers for juveniles, when compared to adults. As a final analysis, we investigated the correlations between infections and three life history characteristics; migratory status, age, and body. The results suggest that age was a significant predictor of overall parasite presence.

ARAS-IĞDIR BÖLGESİNDEKİ GÖÇMEN KUŞLARDA SITMA BULUNMA SIKLIĞI

Kuş sıtma parazitleri tüm dünyaya yayılmıştır ve kuş türlerini geniş bir yelpazede etkiler. Kuş sıtmasına sebep olan *Plasmodium* ve *Haemoproteus*, dünyadaki parazit konak-vektör etkileşimlerinin ekolojik ve evrim çalışmasını yapmak için popüler modellerdir. Kuş sıtması üzerine yapılan çalışmalar daha çok konak kuşlar ve sıtma paraziti üzerine odaklanmıştır. Kuş sıtması aynı zamanda endemik ve göçmen kuşlar arasında yaygın bir hastalıktır. Sıtma enfeksiyonu hızlı populasyon düşüşlerine veya çevresel değişiklikler ve insan faaliyetleri sonucu türlerin yok oluşlarına neden olabilir. Kuş sıtmasının parazit çeşitliliği, Aras-Iğdır bölgesinde bulunan konak populasyonun dağılım ve yaygınlığı gibi belirli yönlerini anlamaya çalışmak, bu tezin ana hedeflerini oluşturmaktadır. Bu amaçlara istinaden *Plasmodium* ve *Haemoprateus* görünme oranları PCR bazlı test ile teyit edilmiştir. Çalışma kapsamında 25 farklı familyadan 58 kuş türüne ait 401 kan örneği incelenmiştir. 15 örnek pozitif olarak belirlenmiş ve 215 bazlık cytochrome b dizisini kapsayan 13 temiz kromatogram, Malavi veritabanından alınan daha önce yayınlanmış 78 *Plasmodium* and 46 *Haemoprateus* PCR yayını ile karşılaştırılmıştır. 7 kuş cinsine ait 13 *Plasmodium* ve *Haemoprateus* dizisi, daha önce yayınlanmamış olan dokuz haplotipe aittir. Bir *Plasmodium* örneği hariç, bütün örnekler Aras-Iğdır bölgesinde yüksek görünme oranına sahip *Haemoprateus*'da kümelenmiştir. Aynı zamanda kuş sıtması enfeksiyonunda yaşa bağlı görünme oranı örüntülerini de inceleyen çalışma sonuçları, hastalıktan kurtulma oranının gençlerde yetişkinlere göre daha fazla olduğu belirlenen daha önceki çalışmalarla tutarlılık göstermiştir. Yapilan son analizde, enfeksiyonlar ile üç yaşamsal karakter olan; göç etme özelliği, yaş ve boy arasındaki ilişki incelenmiştir. Sonuçlar, yaşın bütün parazitlerin olusumunda belirleyici bir etken olduğunu göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols	Explanation
BDNF	Brain-derived Neurotrophic Factor
Bp	Base Pair
CR	Critically Endangered
DNA	Deoxyribonucleic acid
DnaSP	DNA Sequence Polymorphism
Dntp	Deoxyribonucleotide
EIDs	Emerging Infectious Diseases
EN	Endangered
Hap	Haplotype
IUCN	International Union for Conservation of Nature
ML	Maximum Likelihood
NJ	Neighbor-joining
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
VU	Vulnerable

1. INTRODUCTION

In recent years, the world has been losing its diversity at an alarming rate. Human population and their needs increasingly lead to a reduction in biotic richness (Wilson, 1988) and conservationists point out to social, political and economic problems as some of the general causes of extinction of many species (Kellert, 1996). The underrated importance of the loss of biodiversity has severe impacts on human and environmental security. The immediate pressures to species include degradation, over-exploitation, pollution, disease, and global climate change, among others. More specifically, destruction of habitat, wildlife trade, over-hunting, and competition with domestic and non-native animals are some of the other reasons that are related to man-made activities, which result in endangerment and/or extinction of species (Ganly, 2007).

The human population is expected to reach around 10 billion by 2050, which will result in the elimination and alteration of natural habitats all over the world. This will have devastating consequences for species diversity. One-quarter of the world's species threatened, and more specifically subspecies and diverse forms lose their internal genetic variability, affecting their adaptability and ultimate survival (Tilman et al., 2001).

Although extinction is assumed to be a natural process and most species go extinct naturally (the so-called background extinction), the current extinction rates are much higher than these background rates (Myers, 1988; Soule, 1996). Concerning the assessment of the world’s species, more species are under threat of extinction than ever before (IUCN, 2014). The International Union for Conservation of Nature (IUCN) publishes The Red List of Threatened Species, which is one of the main sources of global conservation status assessments for plants and animals. The IUCN emphasizes the risk of extinction for species and develops potential protection methods. In addition, the IUCN prepares a set of levels. Some recent numbers of Critically Endangered (CR), Endangered (EN) and Vulnerable (VU) species on the IUCN Red List, as of 2014 are as follows:

- Total number of threatened animal species was 11,818;
- There are 7,487 recently described vertebrates (mammals, birds, reptiles, amphibians, fishes) that are categorized as under threat;

- The number of extinct bird species was 145;

Conservation biology is a practical and result-driven discipline with the aim of preventing or decreasing the current extinction threat on many biological species. In conservation, it is important to understand the reasons of extinction and endangerment for individual species to muster effective conservation strategies (Clark, 1996). That is to say, extinction is a complex issue and all precautions must be taken on large temporal and geographic scales in order to try to prevent it. If one does not think proactively about extinction rates, it will take many years for speciation to recover the destroyed biodiversity (Millennium Ecosystem Assessment, 2005; Clark, 1996).

1.1. Conservation Status of Vertebrates and Birds in the World

Vertebrates are a group of animals that have five classes and include birds, mammals, reptiles, amphibians and fishes. For conservation purposes, today, vertebrates have been investigated more thoroughly than any other groups and have crucial roles in decision making processes. Some of these reasons include;

- Most of the information about the conservation of countries' biodiversity has been originated and dispersed by this taxonomic group;
- Most species conservation and survival strategies are bound up with vertebrates;
- Vertebrates are one of the most fascinating groups for the public;

Birds are one of the major groups of vertebrates and they are descendants of the reptiles. They exhibit some structural alterations for flight, although not all birds have these modifications. They comprise a significant portion of the global vertebrate diversity. They are also barometers for change in the wider environment in an ecological context. They cover many trophic levels from mid-level consumers to top predators, and even relating to other organisms after death, avians maintain the interaction with their prey and predator species to obtain critical resources for scavengers and decomposers.

Although extinction is a difficult process to document for animals, this has probably been done better for birds than other vertebrate classes. In total, 129 bird species are known to have gone extinct since 1500s. Even in the beginning of the 21st century, four

species have been classified as extinct in the wild. 14 additional species have been recorded as Critically Endangered, and one as 'Critically Endangered possibly extinct in the wild' (Butchart et al. 2006). Areas in the southern hemisphere, with high levels of endemism that are rich in species and in higher taxa have higher numbers of threatened species. More threatened bird species live in tropical areas because these areas exhibit higher levels of threatening processes (Blackburn et al. 1996).

Looking into the 21st century, climate change is also expected to have negative effects on birds. Jetz et al. (2007) stated that climate change and habitat destruction threaten about 10-20% of bird species with extinction by 2100. These researchers also suggested that in addition to the effects of global warming, due to human population growth, 8750 species of terrestrial birds are under threat, and about 950 to 1800 species could go extinct by 2100. In addition, Bonneaud et al. (2009) and Chasar et al. (2009) stated that external effects such as urbanization related to human activity, agricultural practices, and internal factors related to the species themselves can also come into play in causing declines in bird populations. Bird parasites are among these internal factors.

1.2. Current Knowledge of Turkey's Bird Fauna

Located at the crossroads between the Balkans, Mediterranean, the Middle East, and Central Asia, Turkey comprises one of the most important bird migration routes of the world (Sekercioglu, 2006; Kirwan et al., 2008). Vegetation, topography, and climatic condition of Turkey have supported a high number of native bird species. This number is also increasing. For instance, in the first five months of 2011, three new species were identified by Turkish birdwatchers in the country, and Turkey's known bird species increased to 468 (Sekercioglu et al., 2011). From a conservation perspective, according to the latest IUCN Red List (2014) 16 bird species, including migratory species, are categorized as threatened in Turkey.

The main factors that contribute to the decline of avifauna in Turkey include environmental pollution, habitat loss and other damaging forces on ecosystems, as illustrated on various occasions. For instance, on the Mediterranean coast, red-backed shrike (*Lanius collurio*), whinchat (*Saxicola rubetra*), yellow wagtail (*Motacilla flava*),

common starling (*Sturnus vulgaris*), Eurasian linnet (*Carduelis canabina*), and corn bunting (*Emberiza calandra*) populations at the Akyatan, Ağyatan, Tuzla and Yumurtalık lagoons have declined approximately 40-fold from 3 million in 1962 to 76,500 in 2007 (Küyük, 2007). Furthermore, threats have resulted in the disappearance of various farmland species such as the common kestrel (*Falco tinninculus*), northern lapwing (*Vanellus vanellus*), European turtle dove (*Streptopelia turtur*), crested lark (*Galerida cristata*), Eurasian skylark (Alauda (*Miliaria calandra*), both in Europe and Turkey (Sekercioglu et al., 2011).

1.3. Bird - Parasite Interactions

Birds can provide important insights on the patterns of infectious diseases because they help to investigate both human-impacted environments and pristine areas. In the last several decades, there were several examples of emerging infectious diseases (EIDs), many of them rooted in Africa. The possible factors affecting transmission of infectious disease also include interactions between abiotic and biotic components. Initially, intrinsic biotic factors are responsible for the abundance of host infection such as genotypic resistance (Westerdahl et al., 2005; Bonneaud et al., 2006; Loiseau et al., 2008), behavior, sex or age (McCurdy et al., 1998; Ots and Horak, 1998). Extrinsic factors, such as urbanization, population growth, development of farmlands, wildlife trade, the loss of biological diversity, and climatic conditions can also affect parasite species diversity and its prevalence.

In addition, studies have shown that habitat change by human impact, particularly deforestation may have a direct effect on diversity and distribution of diseases (Taylor, 1997). Conversion of forested areas by cutting and clearing, increase in open space areas for access of sunlight (Yasuoka and Levins, 2007) have resulted in an increase in the occurrence of vectors. Thus, the expansion of the vector communities can alter the distribution of bird populations and their common genetic diversity. In this case, the theory indicates that deforestation can indeed cause large-scale transmission of infectious diseases among bird species (Tompkins et al., 2006).

Birds can be exposed to internal and external parasites such as Nematodes, Trematodes, and Cestodes (flukes and tapeworms), Protozoans and Arthropods (Rausch, 1983). Nematodes are the most common parasites that are found in the body system of birds, especially in the intestinal tract. Diagnostic signs include worms appearing in the feces (Cooper 2002, Heidenreich 1997), and their presence can be inferred by an examination of fecal material under the microscope. Parasites such as Trematodes and Cestodes have life cycles that affect birds indirectly. Their life cycles need an intermediate host such as snails and earthworms. Protozoans, another group of parasites, contain Coccidia. Coccidia are blood parasites and the cause blood-tinged feces and diarrhea. In addition, arthropods are another group of bird parasites, which infect skin or feather and respiratory tract of birds. These types of parasites include mites, ticks and flies (Krone, 2007).

A number of blood parasites can also infect birds. These parasites can exert important selection pressure on their hosts through energy investment periods such as migration and reproduction (Remple 2004, Garvin et al. 2006). The rate of infection is higher in migratory birds than nonimmigrants (Moller and Erritzoe 1998). Taking migrants into consideration, migration seems to be a stress condition, and that might be why birds face a high probability of parasitic infections during migrations (Moller and Erritzoe 1998, Smith et al. 2004, Valkiunas 2005).

1.4. Haemosporidian Parasites of Avian Species

Birds host a wide variety of blood parasites. The phylum Apicomplexa is a large community of protozoan parasites, including haemospororin, and can severely infect a great number of classes of vertebrates, resulting in chronic infections during energy demanding periods of wild birds (Merino et al.,2000, Marzal et al. 2005; Knowles et al. 2010). One of the most important types of parasite include those causing malaria, a devastating disease that affects humans and a large number of other mammals, birds and reptiles (Cook, 1971; Olsen, 1974). There are similarities and dissimilarities among mammals and birds in terms of their malaria parasites. Different haemosporidian parasites of birds and mammals undergo several processes inside their hosts' erythrocytes. The nucleated erythrocytes that are common in birds and non-nucleated erythrocytes that are

common in mammals have fundamental differences that separate them into two classes. However, they share the same life cycle and similar dipteran vectors (Cumings et al., 2010).

Avian malaria parasites including *Plasmodium* and related Haemosporidian have significance in ecological and evolutionary studies. Protozoan subclass of Haemosporidia is a large group of vector-borne intracellular parasites. The avian haemosporidian parasites belong to different taxonomic groups (Valkiunas, 2005). There are more than 200 described species classified into *Plasmodium*, *Haemoproteus* and *Leucocytozoon* (Martinsen et al., 2008). Since 2000, about 800 unique cytochrome-b lineages in three genera of avian blood parasites have been collected in GenBank. Studies indicated about 40 different *Plasmodium* species, 130 species of *Haemoproteus* and 35 distinct species of *Leucocytozoon* (Valkiunas, 2005). These three genera of parasites are placed into different taxa, based on their blood-feeding vectors and habitats. The parasites are also transmitted by different families: *Haemoproteus spp.* by biting midges (Ceratopogonidae) and hippoboscid flies (Hippoboscidae), *Plasmodium spp.* by mosquitoes (Culicidae) and *Leucocytozoon spp.* by blackflies (Simuliidae) (Valkiunas, 2005).

Avian haematozoan parasites in the genera *Plasmodium* and *Haemoproteus* are widespread and have been detected in many avian species. Two genera differ in their life cycles and primary vectors. *Haemoproteus* passes through an asexual reproduction phase in various tissues of the vertebrate host before infesting the blood and undergoes both asexual and sexual reproduction in the vector (Atkinson et al., 2009). On the other hand, *Plasmodium* undergoes asexual reproduction in the peripheral blood. For this reason, *Plasmodium* is considered to be more dangerous for their hosts in relation to infection (Van Riper, 1986; Atkinson, 2000).

1.5. The importance of Turkey and Aras River, Iğdır for Bird Migration

Bird migration is a good indicator in evaluating the natural conditions, such as habitat and environmental quality, in a given area. For seasonal migration of birds to be successful, environmental stability and predictability are important factors. In this regard, environmental instability is a negative effect, like the temperature changes between the day

and night in the dry woodland and savannas found in Africa (Alerstam et al., 1979). In order to cope with such instabilities, such as seasonal water shortage in the summer, some bird species undertake long migrations between Europe and Africa, to survive and increase their reproductive success.

Some areas in Turkey, especially those like Aras-Iğdır, are of crucial importance for many migratory bird species. In this respect, Aras River lies at a critical spot on the bird migration path between Eastern Europe and Africa. Aras River, located in the city of Iğdır, is a Ramsar Site, so it is an important stopover area for migratory birds to reproduce. In autumn and spring months, more than 20,000 migratory birds both rest and feed at Turkey's Kars and Igdir provinces. Both stations have over 225 bird species, exceeding 300 species in total (KuzeyDoga Society, 2014).

1.6. Thesis Objective

As avian malaria is an important disease that can affect the survival of bird species, and Aras-Iğdır being an important migratory hotspot, the prevalence of malaria in birds of this area was assessed in this study. For this purpose, *Plasmodium* and *Haemoprateus* cyt b DNA gene was amplified to diagnose malaria positive individuals, and the amplified sequences were combined with sequence data from databases for phylogenetic inference. The life history chracteristics of age and sex were also used to see patterns, if any, in the individuals tested positive and negative for *Plasmodium*/*Haemoprateus*. Phylogenetic tree methods were also applied to evaluate host-specialization patterns and to determine whether host-switching occurs.

2. LITERATURE REVIEW

The advent of polymerase chain reaction (PCR), microscopic investigations and the use of serological techniques have led to the emergence of new research areas based on evolution, phylogeography and phylogenetics of avian malaria parasites. Microscopic examination of blood smears has been traditionally used for parasite detection. In this method, positive results can be detected by observing the desired parasites in Giemsa-stained blood smears (Kirkpatrick and Smith, 1988; Payne, 1988). However, the development of molecular methods, which were observed to be more powerful than microscopy at low levels of parasitemia, increased the popularity of the former. It should be noted that blood smears are still used today, as both quantitative and qualitative assessments can be made with this method.

Although molecular methods are quite useful in detecting parasitemia, they have some disadvantages (e.g. the risk of infection that may be due to contamination in the laboratory environment). On the other hand, molecular techniques are more effective than blood smears as they are faster, cheaper and more reliable, and as they can be optimized (Richard et al., 2002). In addition, the genetic techniques have a powerful sensitivity for identifying and screening the frequency of parasites, with polymerase chain reaction (PCR). For this reason, PCR assays are now being routinely used to detect the avian malaria infections including both *Plasmodium* and *Haemoproteus*. For PCR assays of malaria parasites, generally primer sets are used to amplify fragments of the cytochrome b and the 18S rRNA subunit gene (Feldman et al., 1995).

Serological technique played an important role in determining higher rates of haemosporidian parasites in avian blood (Atkinson, Dusek et al., 2001) and it was understood that serology is more sensitive than PCR (Jarvi et al., 2002). This demonstrates that serological methods are more effective to detect antibodies, which are produced against malaria rather than detecting the actual parasites; on the other hand PCR or microscopic examination cannot be used for antibody detection (Atkinson and Van *Riper*, 1991; Atkinson, Dusek et al., 2001; Atkinson, Lease et al., 2001; Jarvi et al., 2002). However, current (active) infections can be detected by microscopic examinations and

polymerase chain reaction. Eventually, PCR and serology have different advantages, but they are also two complementary techniques that can be used simultaneously to obtain more precise results.

Focusing on the genetics studies further, Li et al. (1995) who identified several conserved regions in the small subunit RNA of *Plasmodium spp.* developed a set of primers that focused exclusively on human strains of parasite. It was assumed that their universal primers would successfully detect a range of *Plasmodium* species, including those that utilize avian hosts. In fact, Perkins et al. (1998) used the same primers to detect malaria infections in the western fence lizard *Sceloporus occidentalis*. More recently, Bensch et al. (2000) obtained results from primers on conserved regions of the cytochrome b (cyt b) region. Although their work focused primarily on building a phylogeny of *Plasmodium* and *Haemoproteus* strains, it also included a comparison between PCR assay and blood smear analyses.

Large numbers of molecular studies were undertaken to describe new *Plasmodium* species in different areas. Valkiunas et al. (2008) detected three new malaria species (*Plasmodium* (*Novyella*) *lucens n. sp.*, *Plasmodium* (*Novyella*) *multivacuolaris n. sp.* and *Plasmodium* (*Novyella*) *parahexamerium n. sp.* which were found in the rainforests of Ghana and Cameroon, and spread from African passeriform birds (respectively found in olive sunbird *Cyanomitra olivacea* (Nectariniidae), yellow-whiskered greenbul *Andropadus latirostris* (Picnonotidae), and white-tailed alethe *Alethe diademata* (Turdidae)).

In Colombia, two new *Plasmodium* species (*Plasmodium vaughani* and *Plasmodium unalis*) parasitizing great thrush (*Turdus fuscater*) were suggested to be phylogenetically sister parasites, but ecologically different species. *Plasmodium vaughani* was later recorded in North America, Europe, Japan, New Zealand, and Turkey (Martinsen et al. 2008; Kim and Tsuda 2010; Glaizot et al. 2012; Howe et al. 2012; Inci et al. 2012; Zehtindjiev et al. 2012b). *P. unalis* was found only in Colombia (Valkiunas et al., 2013).

Geographical distribution has considerable effect on avian malaria. Many studies have supported the variation in malaria prevalence due to variation at regional, temporal and

spatial levels. In one study (Fallon et al., 2003) four of the most abundant and widespread passerine birds in the Lesser Antilles ((*Coereba flaveola* (Bananaquit), *Loxigilla noctis* (Lesser Antillean Bullfinch), *Tiaris bicolor* (Black-faced Grassquit), and *Vireo altiloquus* (Black-whiskered Vireo)), were analyzed considering the effect of the islands. The study suggested that parasite lineages did not vary over the geographic ranges of the hosts, and the alteration of the prevalence of parasites was observed when the host individuals moved between islands. This pattern affected and changed gene flow between these four species and genomic heterogeneity was observed across the islands.

In another study, Hawaiian honeycreepers (Drepanidinae) were studied and *Plasmodium relictum* was detected in presence of thermal and altitudinal constrains. The results suggested that *P. relictum* played a major role in the decline and extinction of native Hawaiian honeycreepers. The estimated data supported that high elevation restricted sporogonic development of avian malaria. Moreover, low environmental temperature decreased the survival of *P. relictum* (Atkinson et al., 2010).

A new lineage of Plasmodium spp. was also recorded in Gansu Province, China. In this study *Parus major* was seen to be infected with the lineage GRW4 (*Plasmodium relictum*) (Zehtindjiev et al., 2013), and was described in sedentary birds in tropical areas of China. The distribution of the lineage extended to African migratory birds. It was suggested that the appearance of this lineage may be a result of a recent host-shift being possible after alterations of the geographical ranges.

Considering the other genus associated with avian malaria, *Haemoproteus*, molecular studies were also conducted to highlight the infection to gain a better understanding of their diversity and host-specificity. In one study, the first microscopic and molecular detection of *Haemoproteus* infection in a Tawny Owl (*Strix aluco*) was reported in Kayseri, Turkey (Yıldırım et al., 2013). According to the study, *Haemoproteus* was relatively more host-specific and restricted to the bird species of the same family, in contrast to *Plasmodium* which showed low host-specificity and was detected in several avian families.

The parasitic prevalence of the *Haemoproteus* and *Plasmodium* was also studied in 79 bird individuals in Peninsular Malaysia (Ivanova et al., 2015). 23 birds were detected as positive for *Haemoproteus* or *Plasmodium* infections, and one individual was recorded as carrying a mixed infection. The results of the investigation showed that infections with parasites of the genus *Haemoproteus* were more predominant compared to those of the genus *Plasmodium*.

In another study, 52 species of wild birds from Eurasia were analyzed with a PCR-based method for a better understanding of the distribution of lineages of Haemosporida. *Haemoproteus minutus, H. pallidus* and *H. pallidulus* were detected as possible agents of haemoproteosis in exotic birds, and infections were especially widespread in the blackbird *Turdus merula* (Palinauskas et al., 2013).

3. MATERIALS AND METHODS

3.1. Aras-Iğdır Map

Aras-Iğdır harbors a globally important bird observation station located in north-eastern Turkey. Its geographical coordinates are 39º24'15"N and 45º21'55" E. The approximate location of Aras-Iğdır is shown below in Figure 3.1.



Figure 3.1. Aras-Iğdır Map.

3.2. DNA Extraction and PCR Amplification

Blood samples of 58 bird species were collected from 401 individual birds. PureLinkTM Genomic DNA Mini Kit (Invitrogen, California) or Roche High Pure PCR Template Preparation Kit (Germany) was used for the DNA extraction. Brain-derived neurotrophic factor (BDNF) primers were used to verify the success of DNA extraction. The set of control primers used were ChickBDNF5' (ATGAC-CATCCTTTTCCTT-ACTATG) and ChickBDNF3' (TCTTCCCCTTTTAATGGTTAATGT-AC). For these PCRs, 25 µl of reaction mixture contained 2 µl of genomic DNA, 0.1 µl of Taq DNA Polymerase (5U/µl), 2 µl of $MgCl_2$

(25mM), 1.25 µl of 10X Taq buffer (Thermo Scientific, Fermentas, Pure Extreme), 0.5 µl of each primer (10 µM), 0.5 µl of dNTPs (10 mM), and 18.65 µl dH_20. The thermal PCR reaction conditions were an initial 5 min. denaturation at 94°C, 35 cycles of 30 sec. denaturation at 94°C, 30 sec. annealing at 55°C, and 30 sec. extension at 72°C, continued by a 7 min. final extension at 72°C.

Avian malaria has been screened for by four different comparative analyses with different primer sets to compare their effectivenes in one published study. They were as follows; the first data (90) and (89) primers , the second two primer sets (570) and (566) - (841) and (844), the third (HAEMF) and (HAEMF2), the final set of primers (621) and (983). According to these comparative analysis of PCR-based detection methods, primer sets 621 and 983 have proven to be far more effective at detecting avian malaria within the samples (Richard et al., 2002). For the detection of avian malaria in our study, a nested PCR procedure was used to amplify the mtDNA cytochrome b gene by using the forward primer621-5'-AAAAATACCCTTCTATCCAAATCT-3', and reverse primer 983-5'- CATCCAATCCATAATAAAGCAT-3'(Richard et al., 2002). The product from the first PCR reaction was used as the template for the second PCR. In both PCR protocols, 50 µl individual reaction mixture contained 47 µl of master mix and 3 µl of genomic DNA. The master mix included 32.75 µl dH_20, 6 µl $MgCl_2$ (25Mm), 5 µl of 10X Taq buffer (Thermo Scientific, Fermentas, Pure Extreme), 1 µl of dNTPs (10mM), 0.25 µl of Taq DNA Polymerase (5U/µl), 1 µl (10 µM) of forward primer (621), and 1 µl (10 µM) of reverse primer (983). The thermal cycling parameters of PCR began with the initial denaturation at 94°C for 5 min., and subsequently, samples were subjected to 35 cycles at 94°C for 30 sec., 48°C annealing for 1 min., and 72°C extension for 1 min.. Final extension was 72°C for 10 min.. The reaction conditions of the second PCR set were same as the first round, and the same cycling profile was applied. All of the PCRs were carried out with both positive and negative controls. A sample was categorized as positive if PCR bands of 350 bp was visualized on a 1.0% agarose gel (Figure 3.2).

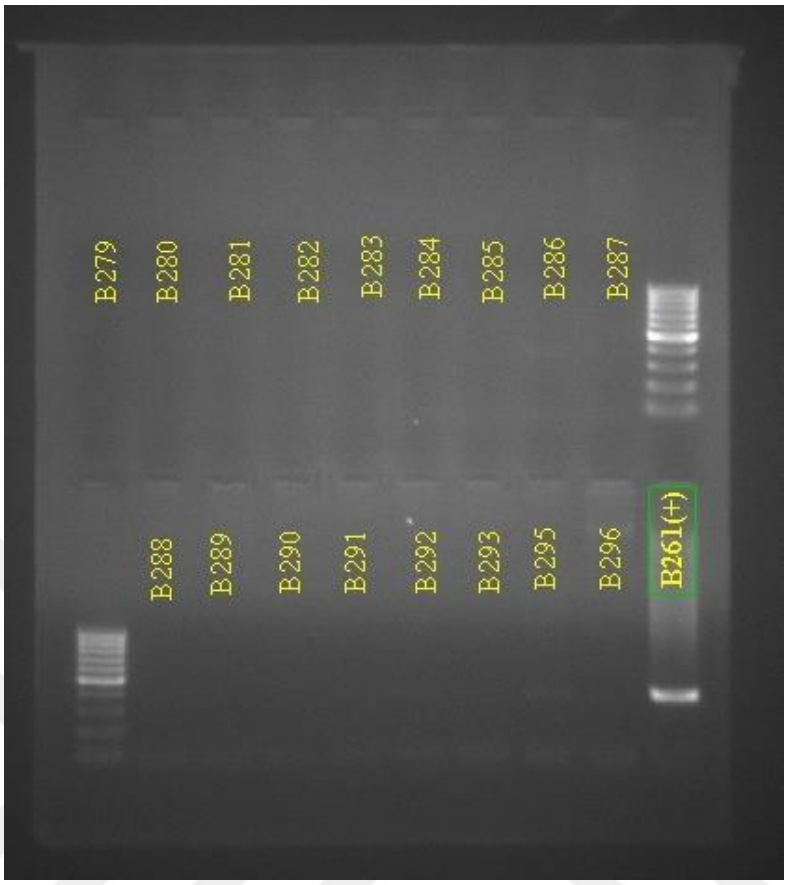


Figure 3.2. The PCR product from the agarose gel amplified by the pair of 621 and 983 primers.

3.3. Sequencing and Alignment

The positive PCR products were commercially sequenced at Macrogen Inc. (Korea), with the forward (621) and reverse PCR primers (983) used for PCR. 13 clean chromatograms of the cytochrome b gene were obtained. Our sequences were compared to 478 cytochrome b sequences of *Plasmodium* and 225 cytochrome b sequences of *Haemoproteus* from Genbank and MalAvi databases (Bensch et al., 2009) The final alignment included 215 bp of 78 *Plasmodium* and 46 *Haemoproteus* sequences, in addition to the 13 sequences of Aras-Iğdır samples. These published DNA sequences and the sequences we obtained from our samples were assembled and aligned using Sequencher v. 5.3. DnaSP v. 5.10 (Librado & Rozas 2009), a software package for the analysis of nucleotide polymorphism from aligned DNA sequence data, was used for the evaluation of the haplotype diversity and the nucleotide diversity.

3.4. Phylogenetic Analyses

In the alignment of sequences, the bases were trimmed at each end of the sequences to match the length of our sequences for the pylogenetic analyses. In addition to our 13 samples, 78 *Plasmodium* and 46 *Haemopratous* seqeunces from databases were used for phylogenetic analyses. Modeltest v 3.7. (Posada, 2010) was used to determine appropriate models of nucleotide substitution, estimated base frequencies, proportion of invariable sites, and a gamma distribution shape parameter. Phylogenetic analyses were performed with the best fitting model of TN93+G(=2.4256)+I (as determined by Modeltest) for maximum likelihood (ML) tree, and the p-distance was used for the neighbor-joining (NJ) tree. For both ML and NJ trees, node support was tested using 1,000 bootstrap replications.

3.5. Malaria prevalence and host traits

A total of 299 birds were captured and tested for presence of parasites. For this analysis we also included information from Akbaba (2012), which showed prevalance levels for the genus *Leucocytozoon* in the same set of bird samples. For our analysis, we removed any birds whose age was undeterminable or whose body weight and wing length were unreliably recorded. This resulted in a sample size of 234 birds 35 of which tested positive for the presence of at least one type of parasite (Table 3.1.)

Table 3.1. Logistic regressions of parasite presence against age status of bird species.

Parasite	Number of birds infected
Leucocytozoon	28
Haemoproteus	3
Leucocytozoon and *Haemoproteus*	3
Leucocytozoon and *Plasmodium*	1

We ran a series of logistic regressions of parasite presence against three predictor variables related to life history of birds: migratory status, age, and body condition. Migratory status was defined as either migrant (found in Aras for only part of each year) or

resident (in Aras year-round). Migratory statuses were assigned to each species using range maps from Collins Bird Guide (Svensson 2009) as well as eBird data. Age was defined as either adult or immature. To determine body condition we performed linear regressions of weight against wing length for each species (Shochat 2002). The regression residuals were then used to predict a bird's mass based on its wing length. Body condition was defined as the ratio of a bird's actual mass to its predicted mass. Regressions were performed using all birds of each species captured in 2009.

We ran three separate logistic regressions with different response variables: 1) Presence of any parasite (not species delineated), 2) Presence of *Leucocytozoon* parasite (regardless of other infections), and 3) Presence of *Haemoproteus* parasite (regardless of other infections). We did not include presence of *Plasmodium* as a response variable as it was only detected in one bird. Similarly, we were unable to include the presence of other parasite species as a predictor variable because so few birds had multiple infections.

4. RESULTS and DISCUSSION

4.1. Determination of *Plasmodium/Haemoproteus spp.* Positive Samples Based on PCR Results

As a result of the control PCR with ChickBDNF primers, 298 samples were detected as positive. Out of these samples, 15 *Plasmodium/Haemoproteus* samples were seen to be positive and were sequenced. In table 4.1, the data are presented for 15 malaria positive samples with their codes, the name of the host species and their collection dates. The number of individuals infected with *Plasmodium/Haemoproteus spp*. was, one for each species of *Acrocephalus arundinaceus, Acrocephalus palustris, Acrocephalus schoenobaenus, Acrocephalus scirpaceus, Carpodacus erythrinus, Locustella luscinioides, Luscinia luscinia, Motacilla flava* and *Phoenicurus phoenicurus*, four individuals for *Passer montanus*, and two individuals for *Phylloscopus trochilus*. Considering the age categories of the positive *Plasmodium/Haemoproteus* samples, five were naive juveniles, three were adults, two were infants and five were undefined. Regarding the sex frequency of the 15 samples, two were female whereas 13 individuals were undefined.

In Table 4.2., baseline data with regards to the prevalence rates are provided. 15 individual birds were infected with malaria and the mean parasite prevalence was 5.0110.8%. The infected individuals belonged to five different families and 11 species. The highest incidence of prevalence was seen in *Passer montanus* of family Passeridae (57.1%). The other infected species in decreasing rates of prevalence were *Phylloscopus trochilus* of family Sylviidae (20%), *Luscinia luscinia* of family Muscicapidae (16.7%), *Acrocephalus schoenobaenus* of family Sylviidae (14.3%), *Locustella luscinioides* of family Sylviidae (14.3%), *Carpodacus erythrinus* of family Fringillidae (14.3%), *Phoenicurus phoenicurus* of family Muscicapidae (8.3%), *Motacilla flava* of family Motacillidae (5.9%), *Acrocephalus arundinaceus* of family Sylviidae (4.8%), *Acrocephalus palustris* of family Sylviidae(3.4%), and *Acrocephalus scirpaceus* of Sylviidae (3.3%). The prevalence rates based on families, from high to low, were 46.7 % of family Sylviidae, 26.7% of family Passeridae, 13.3% of family Muscicapidae, and 6.7% of family Motacillidae and of Fringillidae

Table 4.1. The host name, code and collection date of samples, which were *Plasmodium/Haemoproteus* spp. positive. Positive samples are underlined on the phylogenetic tree (see Figure 4.1. and discussion below).

Name of Species	Code of Sample	Age	Sex	Collection Date
AC ARU(*Acrocephalus arundinaceus*)	FA06744 / B03			31.05.2009
AC RIS(*Acrocephalus palustris*)	JB23215 / B24	N		31.05.2009
AC SCH(*Acrocephalus schoenobaenus*)	JB 23729 / B56			31.08.2009
AC SCI(*Acrocephalus scirpaceus*)	JB23525 / B74	A		26.08.2009
CA ERY(*Carpodacus erythrinus*)	JB 23219 / B99			01.06.2009
LO LUS(*Locustella luscinioides*)	JB 24024 / B198			14.09.2009
LU LUS(*Luscinia luscinia*)	HA 15717 /B201	I		22.08.2009
MO FLA(*Motacilla flava*)	JB 24110 / B236	A	F	09.09.2009
PA MON(*Passer montanus*)	JB 23303 / B261	N		14.08.2009
PA MON(*Passer montanus*)	JB 23465 / B262			22.08.2009
PA MON(*Passer montanus*)	JB 23456 / B263	N		21.08.2009
PA MON(*Passer montanus*)	JB 24019 / B266	N		05.09.2009
PH LUS(*Phylloscopus trochilus*)	RA 26361 / B291	I		14.09.2009
PH LUS(*Phylloscopus trochilus*)	RA 26363 / B292	A		15.09.2009
PH PHO(*Phoenicurus phoenicurus*)	JB 23549 / B295	N	F	28.08.2009

Table 4.2. Evaluation of PCR and sequencing data results according to the prevalence of malaria and the number of individuals and infections. Under “Number of infections”, H and P represent *Hameoproteus* and *Plasmodium* infections, respectively, see Figure 4.1. below.

Order	Family	Species	Number of individuals	Number of infections	Prevalence %
Passeriformes	Sylviidae	*Acrocephalus agricola*	2		
		Acrocephalus arundinaceus	21	1H	4.8
		Acrocephalus palustris	29	1H	3.4
		Acrocephalus schoenobaenus	7	1	14.3
		Acrocephalus scirpaceus	30	1H	3.3
		Cettia cetti	17		
		Hippolais pallida	1		
		Locustella fluviatilis	1		
		Locustella luscinioides	7	1P	14.3
		Phylloscopus collybita	16		
		Phylloscopus lorenzii	1		
		Phylloscopus trochilus	10	2H	20
		Phylloscopus sibilatrix	1		
		Sylvia atricapilla	5		
		Sylvia borin	36		
		Sylvia communis	23		
		Sylvia curruca	5		

Table 4.2. Continued

Order	Family	Species	Number of individuals	Number of infections	Prevalence %
		Sylvia nisoria	1		
	Motacillidae	*Anthus trivialis*	1		
		Motacilla flava	17	1	5.9
	Fringillidae	*Carpodacus erythrinus*	7	1H	14.3
	Emberizidae	*Emberiza citrinella*	1		
		Emberiza hortulana	3		
		Emberiza schoeniclus	2		
		Miliaria calandra	1		
	Muscicapidae	*Erithacus rubecula*	7		
		Ficedula parva	2		
		Luscinia luscinia	6	1H	16.7
		Luscinia svecica	10		
		Muscicapa striata	2		
		Oenanthe hispanica	1		
		Phoenicurus phoenicurus	12	1H	8.3
		Saxicola Maura	1		
		Saxicola rubetra	6		
	Alaudidae	*Galerida cristata*	1		
	Hirundinidae	*Hirundo rustica*	16		
		Riparia riparia	6		
	Laniidae	*Lanius collurio*	17		
		Lanius minor	1		
	Oriolidae	*Oriolus oriolus*	2		
	Passeridae	*Passer domesticus*	5		
		Passer montanus	7	4H	57,1
	Paridae	*Parus major*	3		
	Corvidae	*Pica pica*	1		

Plasmodium prevalence in migratory birds was observed to be low in Aras-Iğdır. Several different hypotheses could be formulated to understand the low estimates of malarial prevalence, including low sensitivity of methods for determination of malaria, resistance to infection, distribution and pathogenicity of parasites and efficient defensive behavior against the vectors.

Some other experimental studies have shown similarly low levels of malaria infection to that recorded in our study. In one example, four captive Omao birds (*Myadestes obscurus*) from the Hawaiian Archipelago were investigated by serological methods to evaluate prevalences of *Plasmodium relictum*. In this study, same dose of malaria was used for both the endemic Hawaiian honeycreepers and *M. obscurus*, which helped to distinguish the parasite susceptibility of the Omao birds. While the native honeycreepers displayed high level of infections, the four captive Omao appeared to have low levels of parasitemia. It was suggested that four individuals of *Myadestes obscurus* have high tolerance to *P. relictum* and developed antibodies to an exposed parasite (Atkinson et. al., 2001).

A similar study was performed with *P. juxtanucleare* in two different regions. In Brazil, chickens exhibited low parasite levels and were susceptible to *P. juxtanucleare* transmissions (Krettli 1972, Silveira et al. 2009a). On the other hand, in Africa, severe clinical signs were seen in chickens during the active malaria (*P. juxtanucleare*) transmission (Grim et al. 2003). The observed differences of prevelance in *P. juxtanucleare* from diverse locations may result from the different immune responses of the hosts to the parasites or different geographical regions discriminating the virulance between parasite lineages.

The low prevalence might be explained by the different stages of the disease as well. The malaria infection usually begins with an acute phase and continues with a chronic phase. The acute stage of the disease tends to display parasitaemia levels with clinical signs, however, symptoms are not expressed in chronic phase of infection (Atkinson & van Riper, 1991). Hence, it is also possible that, the PCRs undertaken were not sensitive enough to detect the malaria infections, if the disease was in a chronic phase (Jarvi et. al., 2002, Durrent et. al., 2006). In one experiment, latent parasites were not detected by the PCR of peripheral blood samples because in the chronic stage of the malaria disease, the parasites are stored in the liver (Valkiunas, 2005). Hence, some other methods such as liver biopsy or histopathology (Cannell et al., 2013) could be sufficient for detection under these circumstances.

As mentioned above, migratory birds are clear candidates for the spread of such parasite infections. Research suggests that pathogen densities and disease may influence the evolution of migratory behavior at the population level (Altizer, 2001). For this reason, the rate of the malaria parasite infections might be higher in migratory birds. However, we detected low prevalence levels in our study, also suggesting that the migratory birds in Aras-Iğdır region might have developed some immunity.

It has also been suggested that the age of individuals can show the signs of parasite infections, and age-dependent patterns of the malaria infections of host populations can indicate the parasite prevalence and disease outbreaks (Wood et. al., 2013). In one study in Africa, the development of parasitemia of the host involving both naturally infected adults and experimentally infected juveniles was monitored (Bensch et. al., 2008). The results showed that the development of parasitemia of native juveniles was higher than that in the adult birds. In our study, for the infected birds for which age category data was available, the results supported previous research; more infections were observed in the native juvenile (n: 5) birds, when compared to adults (n: 3), although sample sizes are not high enough for a statistical comparison.

Aras-Iğdır region is part of an important bird migration route globally, and is a significant stopover site for birds migrating between Africa and Europe. In a study in Nigeria, where *Plasmodium* and *Haemoproteus* infections in 350 individuals of African resident and European migratory songbird species were tested, host sharing was 44% in the haemospiridian lineages (Atkinson & van Riper 1991). The host specificity pattern of our study was comparable to that observed by Atkinson & van Riper (1991): we observed host-switching in one (Sylviidae) of the five families seen to be positive among 25 families investigated in Aras-Iğdır, resulting in a prevalence rate of 20%. The results from both studies demonstrated that host-sharing was common.

4.2. Results of Phylogenetic Analysis

A partial cytochrome b fragment was succesfully amplified and sequenced in 13 samples. The codes and species names of these *Plasmodium/Haemoproteus* sequences are as follows: B03 (*Acrocephalus arundinaceus*), B24 (*Acrocephalus palustris*), B74

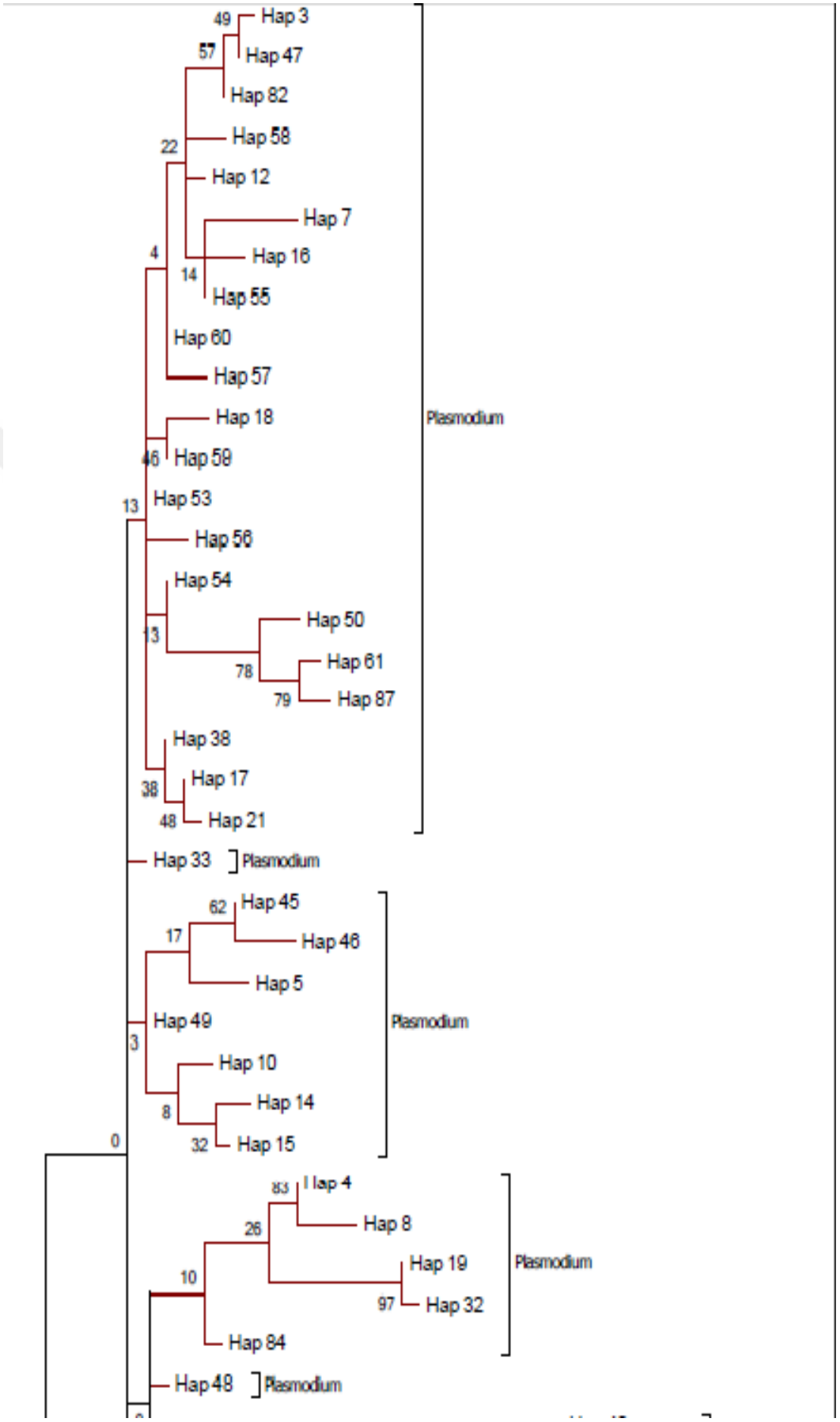
(*Acrocephalus scirpaceus*), B99 (*Carpodacus erythrinus*), B198 (*Locustella luscinioides*), B201 (*Luscinia luscinia*), B295 (*Phoenicurus phoenicurus*), B292 (*Phylloscopus trochilus*), B291 (*Phylloscopus trochilus*), B266 (*Passer montanus*), B263 (*Passer montanus*), B261 (*Passer montanus*), and B262 (*Passer montanus*).

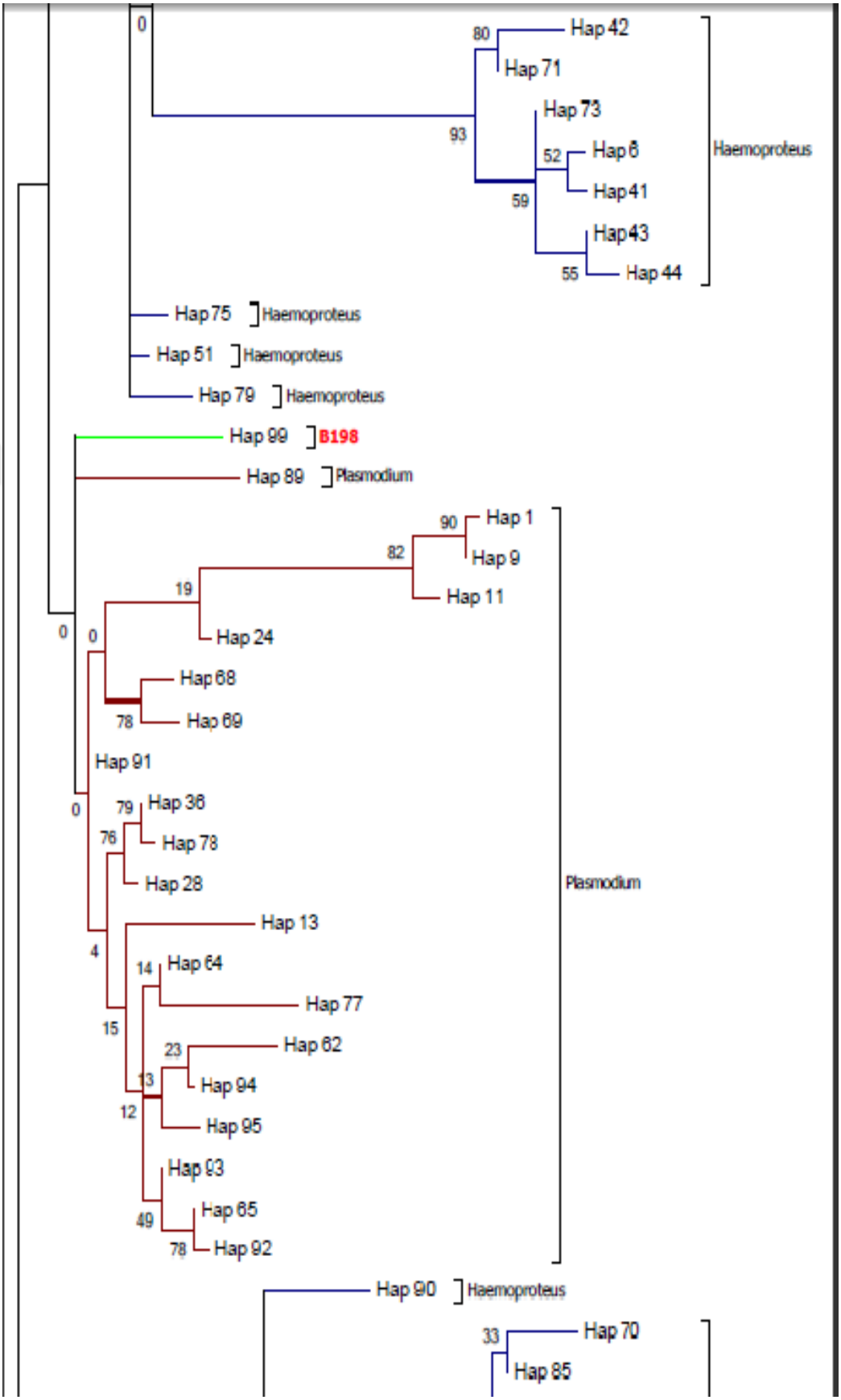
Using the alignment constructed as described below, the maximum likelihood (Figure 4.1) and neigbor-joining trees (Figure 4.2) were seen to have similar topologies. In the whole tree, *Plasmodium* and *Hameoproteus* sequences clustered into two main groups, however some *Hameoproteus* were also seen to cluster with *Plasmodium*. Considering the 13 sequences we obtained in this study, only one (B198, detected in *Locustella luscinioides*) clustered with *Plasmodium*, the other 12 samples clustered with the *Hameoproteus* clade. These 12 samples detected in phylogenetic tree were seen in one individual each of *Acrocephalus arundinaceus, Acrocephalus palustris, Acrocephalus scirpaceus, Carpodacus erythrinus, Luscinia luscinia,* and *Phoenicurus phoenicurus*, two individuals of *Phylloscopus trochilus*, and four individuals of *Passer montanus*. Looking at the haplotypes associated with these host species, we can detect some other patterns. B201 (*Luscinia luscinia*) was seen to cluster together with Hap 34, with a partial sequence from Genbank (AF465587-*Copsychus malabaricus*). B24 (*Acrocephalus palustris*) and B74 (*Acrocephalus scirpaceus*) were also placed closely in the tree, within the *Hameoproteus* clade. B295 (*Phoenicurus phoenicurus*) and B03 (*Acrocephalus arundinaceus*), which comprised the same haplotype (Hap 96) were seen to be observed in different hosts, showing sharing of this haplotype by different hosts. In addition, B261 (*Passer montanus*), B262 (*Passer montanus*), B263 (*Passer montanus*) and B266 (*Passer montanus*) shared the same haplotype (Hap 97), suggesting the specificity of this haplotype to *Passer montanus*. In this last case, *Haemopretous* was observed to be host-specific or at least nearly so, and suggest that it might have its own *Haemoproteus* parasite.

4.3. Variation in parasite prevalence with respect to life history traits

Considering the correlation between parasite infection and the three life historu variables investigated (age, migratory status, and body condition), age was seen as a significant predictor of overall parasite presence (X2, Dev. Res.=4.55, *P*=0.033) as well as the presence of the *Leucocytozoon* parasite specifically (X2, Dev. Res.=4.094, *P*=0.043).

In both cases adult birds showed a significantly greater chance of infection than did immature birds. Age also showed the greatest degree of predictive capability in *Haemoproteus* infection (compared with the other predictors), but was not statistically significant (X2, Dev. Res.=2.83, *P*=0.093). Neither migratory status nor body condition showed any predictive power for any response variable.


49
Hap 3
57
Hap 47
Hap 82
Hap 58
22
Hap 12
Hap 7
Hap 16
4
14
Hap 55
Hap 60
Hap 57
Hap 18
Plasmodium
46
Hap 59
13
Hap 53
Hap 56
Hap 54
Hap 50
13
78
Hap 61
79
Hap 87
Hap 38
Hap 17
38
48
Hap 21
Hap 33
Plasmodium
62
Hap 45
17
Hap 46
Hap 5
Hap 49
Plasmodium
3
Hap 10
Hap 14
8
0
32
Hap 15
83
Hap 4
Hap 8
26
Hap 19
Plasmodium
10
97
Hap 32
Hap 84
Hap 48
Plasmodium


Hap 42
Hap 71
Hap 73
Hap 6
Hap 41
Hap 43
Hap 44
Haemoproteus
Hap 75
Haemoproteus
Hap 51
Haemoproteus
Hap 79
Haemoproteus
Hap 99
B198
Hap 89
Plasmodium
Hap 1
Hap 9
Hap 11
Hap 24
Hap 68
Hap 69
Hap 91
Hap 36
Hap 78
Hap 28
Hap 13
Hap 64
Hap 77
Hap 62
Hap 94
Hap 95
Hap 93
Hap 65
Hap 92
Plasmodium
Hap 90
Haemoproteus
Hap 70
Hap 85

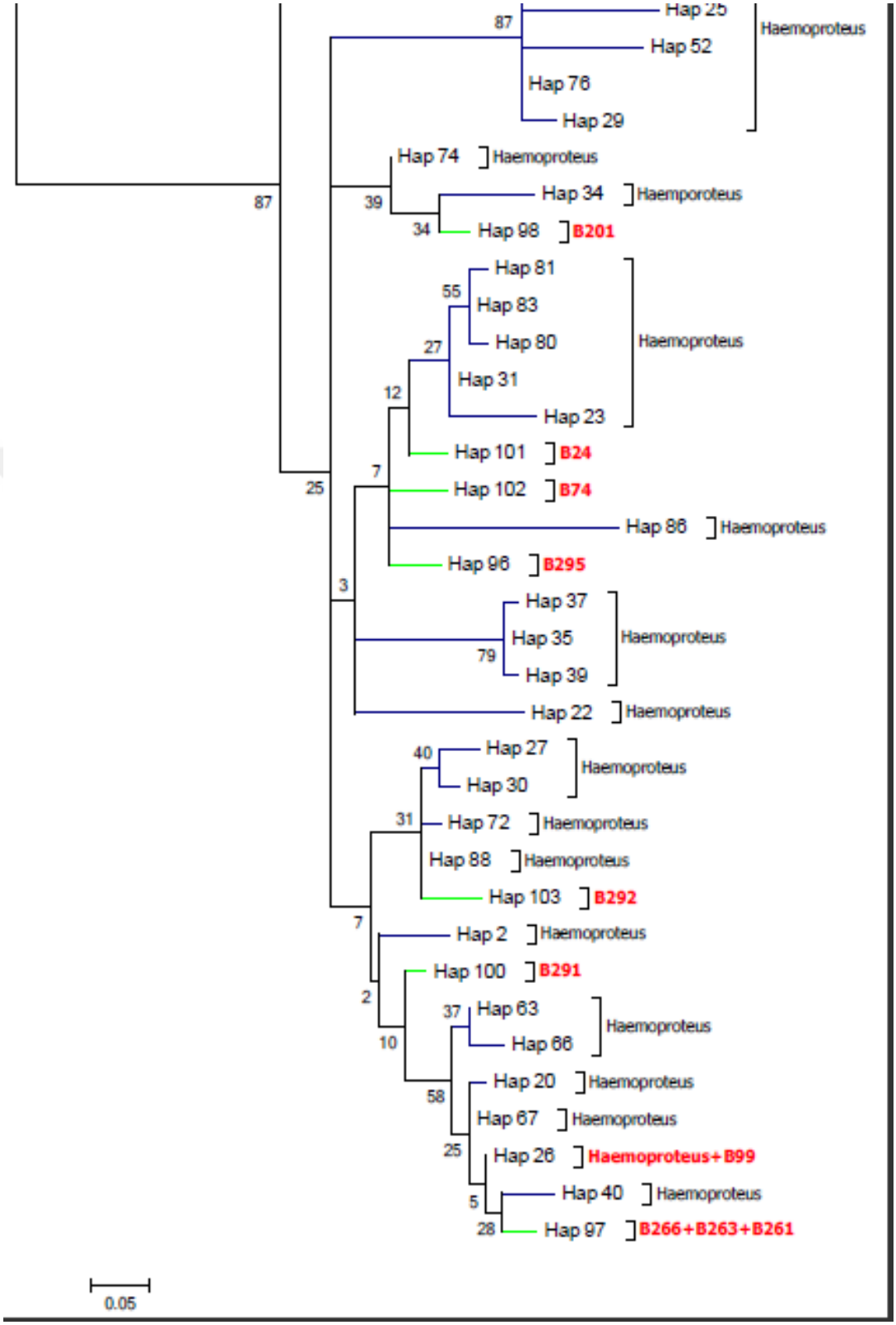
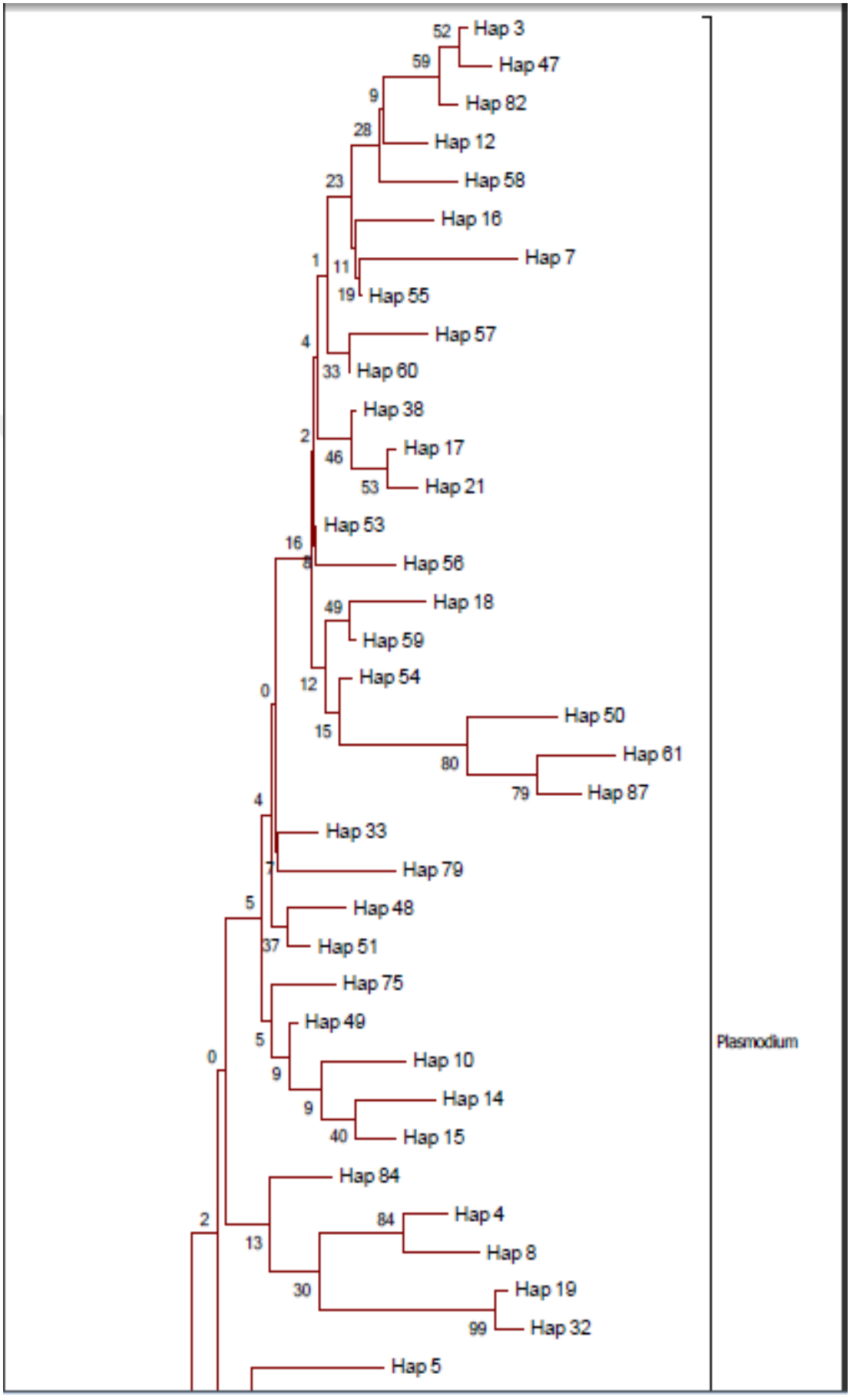
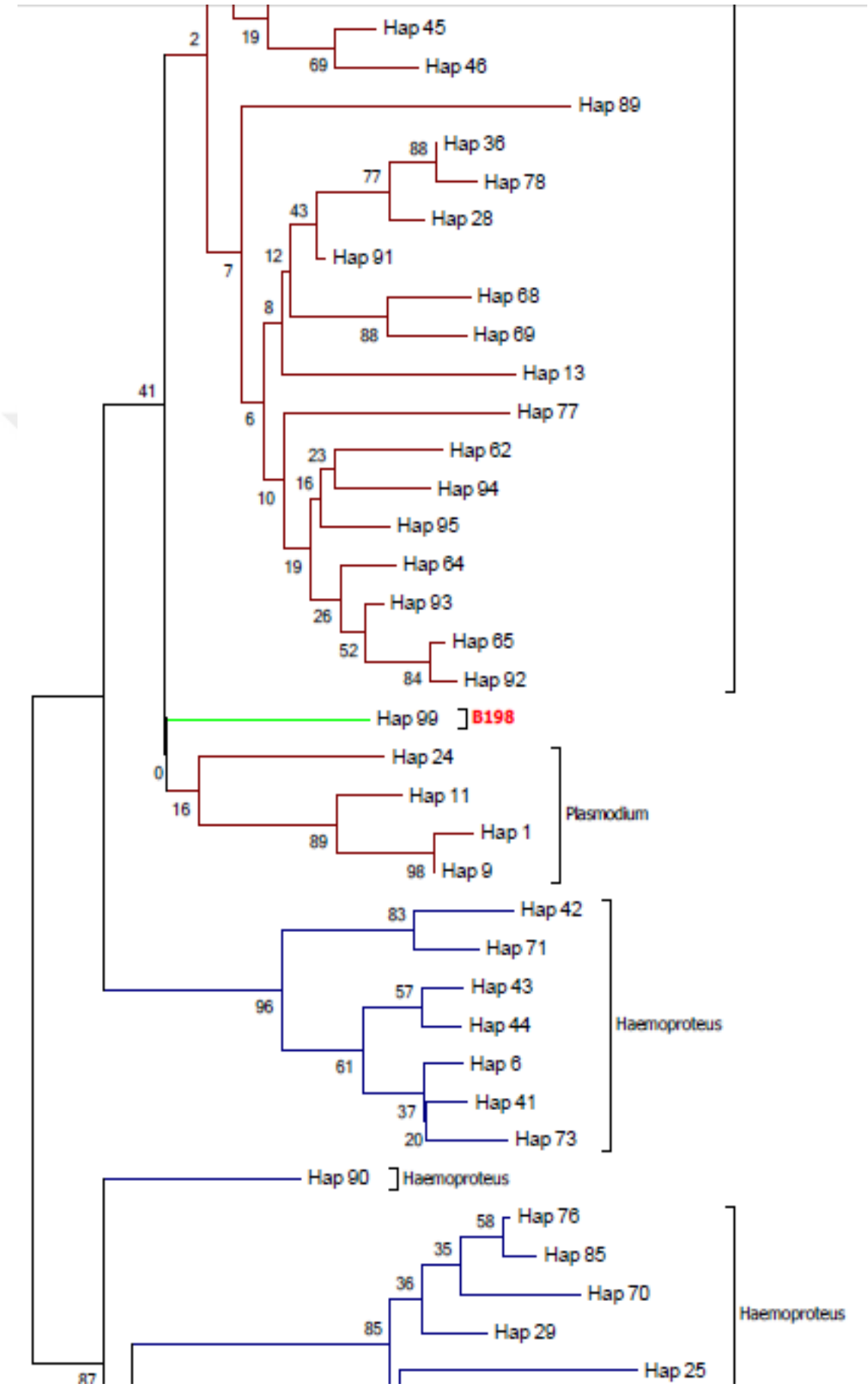


Figure 4.1. Phylogenetic relationships of 103 haplotypes of Plasmodium and Haemoproteus parasites based on mitochondrial cytochrome b gene, constructed using maximum-likelihood method. The sequences obtained in this study are shown in red.


Hap 3
Hap 47
Hap 82
Hap 12
Hap 58
Hap 16
Hap 7
Hap 55
Hap 57
Hap 60
Hap 38
Hap 17
Hap 21
Hap 53
Hap 56
Hap 18
Hap 59
Hap 54
Hap 50
Hap 61
Hap 87
Hap 33
Hap 79
Hap 48
Hap 51
Hap 75
Hap 49
Hap 10
Hap 14
Hap 15
Hap 84
Hap 4
Hap 8
Hap 19
Hap 32
Hap 5
Plasmodium


Hap 45
Hap 46
Hap 89
Hap 36
Hap 78
Hap 28
Hap 91
Hap 68
Hap 69
Hap 13
Hap 77
Hap 62
Hap 94
Hap 95
Hap 64
Hap 93
Hap 65
Hap 92
Hap 99
B198
Hap 24
Hap 11
Hap 1
Hap 9
Plasmodium
Hap 42
Hap 71
Hap 43
Hap 44
Hap 6
Hap 41
Hap 73
Haemoproteus
Hap 90
Haemoproteus
Hap 76
Hap 85
Hap 70
Hap 29
Hap 25
Haemoproteus

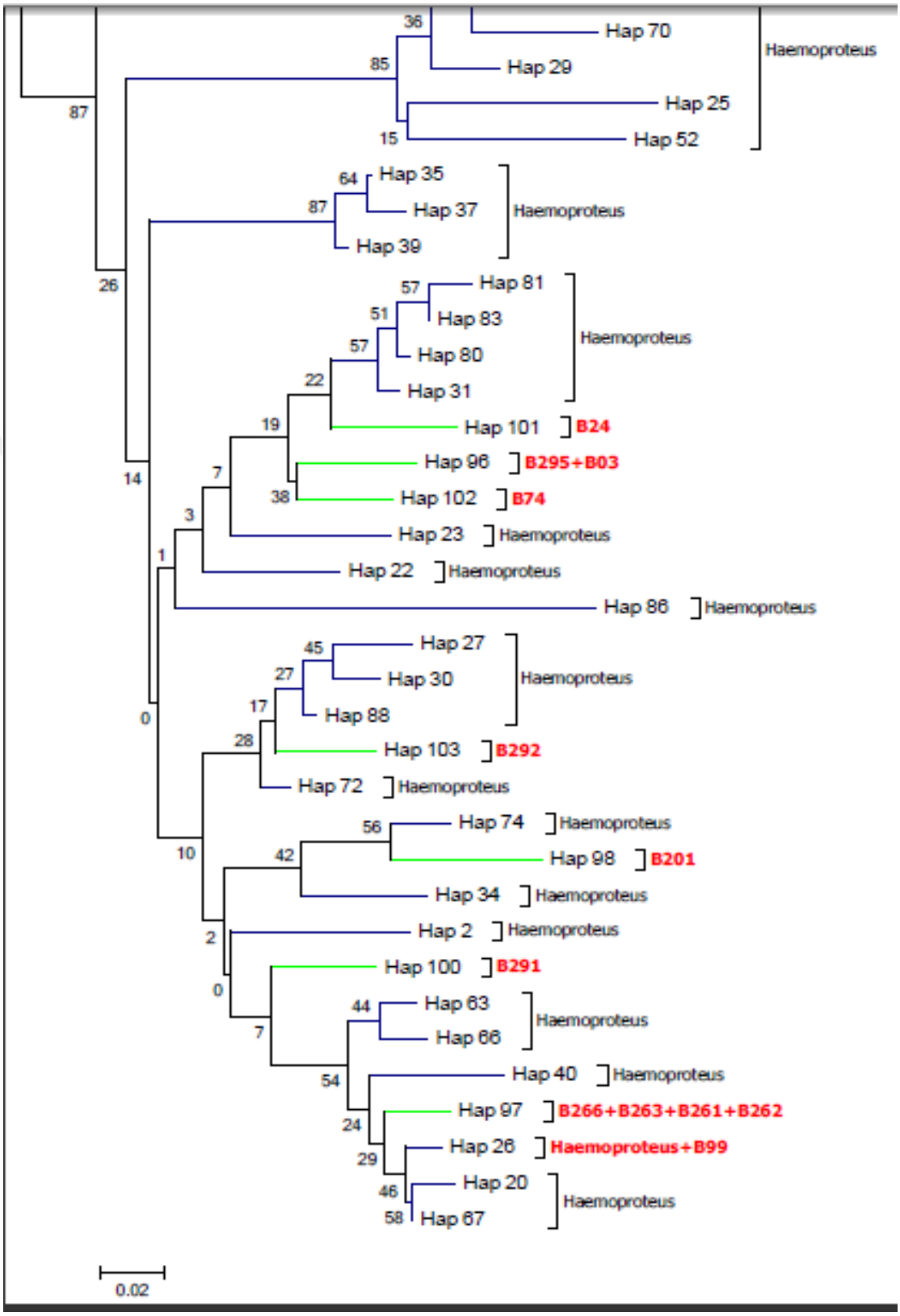


Figure 4.2. Phylogenetic relationships of 103 haplotypes of Plasmodium and Haemoproteus parasites based on mitochondrial cytochrome b gene, constructed using neigbor-joining method. The sequences obtained in this study are shown in red.

5. CONCLUSION AND RECOMMENDATIONS

The study presented here investigated how the prevalence of malaria is influenced by geographical factors, genetic and morphologic traits of parasites, and finally host-parasite interactions. The results indicate that the prevalence of malaria in the migratory birds of Aras-Iğdır region were lower than expected, which could be due to the stage of infection, so that the chronic stage of malaria infection observed would have led to a low probability of infection, and/or the immune response of the host which plays a significant role in determining the prevalence of *Plasmodium* and *Haemoproteus*. Second, age-related patterns in avian malaria infections were consistent with the previous studies, which showed that disease survival rates were high for juveniles compared to adults. As also among the host characteristics. age was found to influence infection rates especially for *Leucocytozoon* parasite.

In this investigation, the avian malaria prevalence and *Plasmodium*/*Haemoproteus* lineage composition was investigated in a single season only (spring). We were not able to estimate the prevalence of malaria in Aras-Iğdır in different seasons, including autumn, which can be undertaken as a follow-up step for this study. In addition, more experimental studies should be made in order to understand how patterns of malaria prevalence are likely to change under future climate conditions.

Finally, complex relationships were discovered between malaria parasites of *Plasmodium* and *Haemoproteus*, and their avian hosts. The phylogenetic tree constructed with 13 unique avian haemosporidian lineages and published species from Malavi database showed that *Haemoproteus* was more prevalent than *Plasmodium* in Aras-Iğdır region, and the possibility of a *Haemoprotean* haplotype specific to *Passer montanus*.

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APPENDIX A: SUMMARY INFORMATION OF BIRD SPECIES BASED ON THEIR AVIAN MALARIA CLADE

Table A.1. Summary information of bird species based on their avian malaria clade (*Plasmodium* or *Haemoproteus*).

Plasmodium	***Haemoproteus***
Aegithina tiphia	*Acrocephalus arundinaceus B03*
Aegolius funereus	*Acrocephalus palustris B24*
Agelaius icterocephalus	*Acrocephalus scirpaceus B74*
Alauda arvensis	*Alcedo leucogaster*
Andropadus latirostris (2)	*Alophoixus bres*
Asio otus	*Asio otus (2)*
Baelophus bicolor (2)	*Carpodacus erythrinus B99*
Carpodacus mexicanus (2)	*Coereba flaveola*
Bubo virginianus	*Columbina passerina socorrensis (3)*
Catharus ustulatus	*Copsychus malabaricus (2)*
Cinclocerthia ruficauda	*Corvus brachyrhynchos*
Cinnyris coquerellii	*Culex pipiens pallens (3)*
Coereba flaveola	*Cyanomitra obscura (4)*
Icteria virens (2)	*Dendrocygna javanica*
Icterus cayanensis	*Dendroica pensylvanica*
Coquillettidia	*Dendroica petechia*
Coquillettidia aurites (2)	*Eudynamys cyanocephala*
Coquillettidia spp.	*Luscinia luscinia B201*
Culex fuscanus	*Passer montanus B261*
Culex pipiens quinquefasciatus (3)	*Passer montanus B266*
Culex vorax	*Phoenicurus phoenicurus B295*
Cyanomitra obscura	*Phylloscopus trochilus B291 (2)*
Cyanomitra olivacea	*Zenaida aurita*
Cyanomitra oritis	
Dendroica discolor	
Dendroica petechia	
Emberizoides herbicola	
Foudia seychellarum	
Gallus gallus	
Geothlypis trichas (2)	
Gyps bengalensis	
Geothylpis trichas	
Linurgus olivaceus	
Locustella luscinioides B198	
Molothrus ater	
Molothrus bonariensis	
Motacilla alba	

Myiarchus tyrannulus	
Nectarinia olivacea	
Niltava sundara	
Ninox scutulata	
Passer melanurus	
Passerina cyanea	
Piranga olivacea	
Ploceus princeps	
Ploceus velatus	
Pomatorhinus ferruginosus	
Pseudoleistes virescens	
Rhipidura rufifrons	
Strix varia	
Sturnella supercilliaris	
Tachycineta bicolor	
Turdus fuscater	
Turdus migratorius	
Zenaida macroura	
Zonotrichia leucophrys	
Zosterops xanthochrous	

APPENDIX B : INFORMATION OF HOST SPECIES AND THEIR HAPLOTYPES

Table B.1. The phylogenetic relationships between the bird species studied were given in Figure 4.1. The haplotypes in the phylogenetic tree, and the host species in which they were recorded, including those from Genbank and Malawi are given in the table below.

Hap1	KC138226 *Turdus fuscater*
Hap2	AY099034 *Vireo olivaceus*, AF465576 *Vireo olivaceus*
Hap3	GQ141593 *Baeolophus bicolor*, DQ659541 *Carpodacus mexicanus*, DQ659539 *Molothrus ater*
Hap4	GQ141580 *Molothrus bonariensis*
Hap5	GQ141560 *Bubo virginianus*
Hap6	GQ141564 *Zenaida aurita*
Hap7	GQ141591 *Coereba flaveola*
Hap8	GQ141582 *Agelaius icterocephalus*
Hap9	AY099032 *Zeneida macroura*
Hap10	AY099035 *Ninox scutulata*
Hap11	GQ141590 *Icteria virens*, GQ141596 *Dendroica discolor*, AF465556 *Passerina cyanea*, AY640145 *Carpodacus mexicanus*
Hap12	GQ141595 *Piranga olivacea*, DQ659547 *Icteria virens*
Hap13	KC771248 *Turdus fuscater*
Hap14	AY099033 *Turdus migratorius*
Hap15	AY099029 *Gallus gallus*
Hap16	AF069611 unpublished, DQ659550 *Emberizoides herbicola*, DQ659548 *Myiarchus tyrannulus*, DQ659551 *Sturnella supercilliaris*
Hap17	AB308044 *Culex pipiens quinquefasciatus*, DQ659565 *Motacilla alba*, DQ659564 *Passer melanurus*
Hap18	AB308046 *Culex fuscanus*
Hap19	AB308052 *Coquillettidia spp.*
Hap20	KF537329 *Zonotrichia capensis*
Hap21	AB308048 *Culex pipiens quinquefasciatus*
Hap22	AB542065 *Culex pipiens pallens*
Hap23	AB542068 *Culex pipiens pallens*
Hap24	AF465549 *Zonotrichia leucophrys*, DQ490064 *Catharus ustulatus*
Hap25	AF465591 *Cygnus columbianus*
Hap26	AF465580 *Dendroica pensylvanica*, AY640142 Carpodacus mexicanus, B99 (*Carpodacus erythrinus*)
Hap27	AF465571 *Alophoixus bres*
Hap28	AF465553 *Cinclocerthia ruficauda*
Hap29	AF465592 *Eudynamys cyanocephala*
Hap30	AF465573 *Corvus brachyrhynchos*
Hap31	AB542067 *Culex pipiens pallens*

Hap32	AB308051 *Culex pipiens quinquefasciatus*
Hap33	EU627835 *Strix varia*
Hap34	AF465587 *Copsychus malabaricus*
Hap35	EU627829 *Copsychus malabaricus*
Hap36	AF465555 *Baelophus bicolor*, AY640128 *Tachycineta bicolor*, AY640134 *Dendroica petechia*
Hap37	EU627830 *Tyto alba*
Hap38	AB474379 *Culex vorax*
Hap39	EU627838 *Tyto alba*
Hap40	AF465578 *Erythrura prasina*
Hap41	JN788936 *Zenaida macroura*
Hap42	JN788935 *Columbina passerina socorrensis*
Hap43	JN788932 *Zenaida macroura*
Hap44	JN788933 *Columbina passerina socorrensis*
Hap45	GQ150187 *Coquillettidia*
Hap46	GQ150188 *Coquillettidia aurites*
Hap47	DQ659542 *Geothlypis trichas*
Hap48	DQ659587 *Cyanomitra olivacea*
Hap49	DQ659581 *Aegithina tiphia*
Hap50	DQ659579 *Cyanomitra olivacea*, FJ404707 *Cyanomitra obscura*, FJ424521 *Cyanomitra obscura*, DQ508392 unpublished, DQ508393 unpublished
Hap51	DQ659585 *Pomatorhinus ferruginosus*, DQ659586 *Niltava sundara*
Hap52	DQ659592 *Alcedo leucogaster*
Hap53	DQ659575 *Ploceus princeps*, DQ659569 *Alauda arvensis*, DQ659560 *Cinnyris coquerellii*, DQ659561 *Foudia seychellarum*
Hap54	DQ659573 *Rhipidura rufifrons*
Hap55	DQ659552 *Alethe diademata*
Hap56	DQ659558 *Ploceus velatus*
Hap57	DQ659567 *Zosterops xanthochrous*
Hap58	DQ659545 *Icterus cayanensis*
Hap59	DQ659554 *Linurgus olivaceus*
Hap60	DQ659568 *Cyanomitra oritis*
Hap61	FJ404705 *Cyanomitra obscura*
Hap62	FJ424524 *Cyanomitra obscura*
Hap63	FJ404699 *Cyanomitra obscura*, FJ404698 *Cyanomitra obscura*
Hap64	FJ404720 *Andropadus latirostris*, DQ241516 *Pseudoleistes virescens*, DQ508385 unpublished
Hap65	FJ404719 *Andropadus latirostris*
Hap66	FJ404696 *Cyanomitra obscura*
Hap67	JN792147 *Catharus ustulatus* (Swainson's thrush)

Hap68	DQ212194 *Gyps tenuirostris*
Hap69	DQ212195 *Gyps bengalensis*
Hap70	DQ212192 *Dendrocygna javanica*
Hap71	JN788939 *Columbina passerina socorrensis*
Hap72	JN792152 *Catharus ustulatus* (Swainson's thrush)
Hap73	JN788940 *Zenaida macroura*, JN788941 *Zenaida macroura*
Hap74	AF465588 *Alethe poliocephala*
Hap75	EU627841 *Aegolius funereus*
Hap76	EU627842 *Asio otus*
Hap77	EU627844 *Asio otus*
Hap78	AY640143 *Geothylpis trichas*
Hap79	EF187495 unpublished, EF187489 unpublished
Hap80	AY640129 *Dendroica petechia*
Hap81	AY640150 *Carpodacus mexicanus*
Hap82	AY640130 *Tachycineta bicolor*
Hap83	AY640133 *Carpodacus mexicanus*
Hap84	AY640137 *Dendroica petechial*
Hap85	EU627836 *Asio otus*
Hap86	FJ404700 *Cyanomitra obscura*
Hap87	FJ404717 *Cyanomitra obscura*
Hap88	DQ490060 *Catharus ustulatus*
Hap89	AF465551 *Nectarinia olivacea*
Hap90	AF465567 *Coereba flaveola*
Hap91	GQ150190 *Coquillettidia aurites*
Hap92	DQ508377 unpublished
Hap93	DQ508379 unpublished
Hap94	DQ508381 unpublished
Hap95	DQ508383 unpublished
Hap96	B295 (*Phoenicurus phoenicurus*) , B03 (*Acrocephalus arundinaceus*)
Hap97	B266, B263, B261, B262 (*Passer montanus*)
Hap98	B201 (*Luscinia luscinia*)
Hap99	B198 (*Locustella luscinioides*)
Hap100	B291 (*Phylloscopus trochilus*)
Hap101	B24 (*Acrocephalus palustris*)
Hap102	B74 (*Acrocephalus scirpaceus*)
Hap103	B292 (*Phylloscopus trochilus*)

APPENDIX C : INFORMATION OF THE SAMPLES USED IN THE STUDY

Table C.1. Information on the field code, species name, age, sex, and collection date for the samples used in the study. The codes of *Plasmodium* and *Haemoprateus* positive samples are highlighted.

Ring Code/Lab Code	Species	Age	Sex	Date of Collection
JB 24031/B01	*Acrocephalus Agricola*	İ		06.09.2009
JB 24015/B02	*Acrocephalus Agricola*	A		05.09.2009
FA06744/B03	*Acrocephalus arundinaceus*			31.05.2009
FA 06746/B04	*Acrocephalus arundinaceus*	N		01.06.2009
FA 06728/B05	*Acrocephalus arundinaceus*	N		31.05.2009
FA 06743/B09	*Acrocephalus arundinaceus*	N		31.05.2009
FA 06800/B10	*Acrocephalus arundinaceus*	İ		18.08.2009
FA 06793/B12	*Acrocephalus arundinaceus*	A		17.08.2009
FA 07439/B13	*Acrocephalus arundinaceus*			23.08.2009
FA 06829/B14	*Acrocephalus arundinaceus*	İ		22.08.2009
FA 06828/B15	*Acrocephalus arundinaceus*	İ		22.08.2009
FA 06787/B16	*Acrocephalus arundinaceus*	İ		15.08.2009
FA 06840/B18	*Acrocephalus arundinaceus*	İ		25.08.2009
FA 07906/B19	*Acrocephalus arundinaceus*	A		13.09.2009
FA 06891/B20	*Acrocephalus arundinaceus*	İ		07.09.2009
FA 07904/B21	*Acrocephalus arundinaceus*	İ		11.09.2009
FA 06898/B22	*Acrocephalus arundinaceus*	a		10.09.2009
JB 23215/B24	*Acrocephalus palustris*	n		31.05.2009
JB 23375/B25	*Acrocephalus palustris*	i		18.08.2009
JB 23441/B26	*Acrocephalus palustris*	i		21.08.2009
JB 23452/B28	*Acrocephalus palustris*	i		21.08.2009
JB 23453/B29	*Acrocephalus palustris*	i		21.08.2009
JB 23363/B30	*Acrocephalus palustris*	i		17.08.2009
JB 23447/B31	*Acrocephalus palustris*	i		21.08.2009
JB 23300/B32	*Acrocephalus palustris*	i		14.08.2009
JB 23472/B33	*Acrocephalus palustris*	i		23.08.2009
JB 23470/B34	*Acrocephalus palustris*	i		23.08.2009
JB 23335/B35	*Acrocephalus palustris*	i		16.08.2009
JB 23471/B36	*Acrocephalus palustris*	i		23.08.2009
JB 23473/B37	*Acrocephalus palustris*	i		23.08.2009
JB 23423/B38	*Acrocephalus palustris*	i		22.08.2009
JB 23440/B39	*Acrocephalus palustris*			21.08.2009
JB 23540/B40	*Acrocephalus palustris*	i		27.08.2009
JB 23589/B41	*Acrocephalus palustris*	i		29.08.2009
JB 23552/B42	*Acrocephalus palustris*	i		28.08.2009
JB 23517/B43	*Acrocephalus palustris*	i		26.08.2009
JB 23521/B44	*Acrocephalus palustris*	i		26.08.2009
JB 23527/B45	*Acrocephalus palustris*	i		26.08.2009
JB 24097/B46	*Acrocephalus palustris*	i		08.09.2009

JB 23528/B47	*Acrocephalus palustris*	i		26.08.2009
JB 23976/B49	*Acrocephalus palustris*	i		03.09.2009
JB 23739/B50	*Acrocephalus palustris*	i		01.09.2009
JB 23554/B54	*Acrocephalus schoenobaenus*	i		28.08.2009
JB 23516/B55	*Acrocephalus schoenobaenus*	i		26.08.2009
JB 23729 /B56	*Acrocephalus schoenobaenus*			31.08.2009
JB 23216/B59	*Acrocephalus scirpaceus*	i		01.06.2009
JB 23309/B62	*Acrocephalus scirpaceus*	a		14.08.2009
JB 23311/B63	*Acrocephalus scirpaceus*	i		15.08.2009
JB 23304/B65	*Acrocephalus scirpaceus*	a		14.08.2009
JB 23479/B66	*Acrocephalus scirpaceus*	i		23.08.2009
JB 23205/B67	*Acrocephalus scirpaceus*	n		31.05.2009
JB 23467/B68	*Acrocephalus scirpaceus*	i		22.08.2009
JB 23333/B69	*Acrocephalus scirpaceus*	n		16.08.2009
JB 23588/B70	*Acrocephalus scirpaceus*	i		29.08.2009
JB 23543/B71	*Acrocephalus palustris*	n		27.08.2009
JB 23529/B72	*Acrocephalus palustris*	n		26.08.2009
JB 23525/B74	*Acrocephalus scirpaceus*	a		26.08.2009
JB 24029/B76	*Acrocephalus scirpaceus*	i		06.09.2009
JB 24043/B77	*Acrocephalus scirpaceus*	i		07.09.2009
JB 24088/B80	*Acrocephalus scirpaceus*	i		08.09.2009
JB 23998/B81	*Acrocephalus scirpaceus*	i		04.09.2009
JB 23590/B82	*Acrocephalus scirpaceus*	i		29.08.2009
JB 24001/B83	*Acrocephalus scirpaceus*	i		04.09.2009
JB 24105/B87	*Acrocephalus scirpaceus*	i		09.09.2009
JB 24131/B89	*Acrocephalus scirpaceus*	i		10.09.2009
JB 23731/B90	*Acrocephalus scirpaceus*	i		31.08.2009
YH 03027/B91	*Alcedo atthis*	a	F	17.08.2009
YH 03042/B92	*Alcedo atthis*	i	F	09.09.2009
YH 03043/B93	*Alcedo atthis*	i	M	09.09.2009
YH 03053/B94	*Alcedo atthis*	i		14.09.2009
JB 24042/B97	*Anthus trivialis*	n		07.09.2009
JB 23218 /B98	*Carpodacus erythrinus*	n		01.06.2009
JB 23219 /B99	*Carpodacus erythrinus*			01.06.2009
JB 23307/B100	*Carpodacus erythrinus*	a	M	15.08.2009
JB 23738/B101	*Carpodacus erythrinus*	i		01.09.2009
JB 24153/B102	*Carpodacus erythrinus*			11.09.2009
JB 24143/B103	*Carpodacus erythrinus*	n		11.09.2009
JB 24142/B104	*Carpodacus erythrinus*	n		10.09.2009
DA 03759/B105	*Caprimulgus europaeus*	a	F	27.08.2009
DA 03762/B106	*Caprimulgus europaeus*	i	M	03.09.2009
DA 03764/B107	*Caprimulgus europaeus*	i	F	04.09.2009
JB 22445/B108	*Cettia cetti*			31.05.2009
JB 23457/B109	*Cettia cetti*	n		22.08.2009
JB 23321/B110	*Cettia cetti*	n		21.08.2009

JB 23236/B111	*Cettia cetti*	n		18.08.2009
JB 22339/B112	*Cettia cetti*	n		31.05.2009
JB 23368/B113	*Cettia cetti*	i	M	15.08.2009
JB 23458/B114	*Cettia cetti*	n		22.08.2009
JB 23249/B115	*Cettia cetti*	n		16.08.2009
JB 02346/B116	*Cettia cetti*	n		08.09.2009
JB 24049/B117	*Cettia cetti*	n		07.09.2009
JB 23256/B118	*Cettia cetti*	n		07.09.2009
JB 12968/B119	*Cettia cetti*	a		12.09.2009
JB 23961/B120	*Cettia cetti*	n		15.09.2009
JB 24108/B121	*Cettia cetti*	n		09.09.2009
JB 24163/B122	*Cettia cetti*	n		12.09.2009
JB 23243/B123	*Cettia cetti*	n		09.09.2009
JB 23321/B124	*Cettia cetti*	n		10.09.2009
CS 00258/B125	*Coturnix coturnix*		F	25.08.2009
CS 00257/B126	*Coturnix coturnix*		F	24.08.2009
CA 00379/B127	*Coracias garrulous*	i		18.08.2009
CA 00372/B128	*Cuculus canorus*	i		16.08.2009
CA 00378/B129	*Cuculus canorus*	i		17.08.2009
HA 15790/B131	*Emberiza citronella*	i	M	24.10.2009
JB 23482/B132	*Emberiza hortulana*	i		24.08.2009
JB 23483/B133	*Emberiza hortulana*	a		24.08.2009
JB 23997/B134	*Emberiza hortulana*	i		04.09.2009
JB 25231/B135	*Emberiza schoeniclus*	a	F	30.10.2009
JB 25226/B136	*Emberiza schoeniclus*	i	F	28.10.2009
JB 25186/B138	*Erithacus rubecula*	i		24.10.2009
JB 25205/B141	*Erithacus rubecula*	i		26.10.2009
JB 25148/B142	*Erithacus rubecula*	i		24.10.2009
FA 06900/B146	*Galerida cristata*	i		11.09.2009
JB 23550/B147	*Hippolais pallida*	i		28.08.2009
JB 23299/B148	*Hirundo rustica*	i		14.08.2009
JB 23362/B149	*Hirundo rustica*	i		17.08.2009
JB 23301/B150	*Hirundo rustica*	i		14.08.2009
JB 23439/B151	*Hirundo rustica*	i		11.08.2009
JB 23338/B152	*Hirundo rustica*	i		16.08.2009
JB 23302/B153	*Hirundo rustica*	i		14.08.2009
JB 23511/B154	*Hirundo rustica*	i		25.08.2009
JB 24074/B155	*Hirundo rustica*	i		07.09.2009
JB 24188/B156	*Hirundo rustica*	i		13.09.2009
JB 24044/B157	*Hirundo rustica*	i		07.09.2009
JB 24047/B158	*Hirundo rustica*	i		07.09.2009
JB 24215/B159	*Hirundo rustica*	i		14.09.2009
JB 23726/B160	*Hirundo rustica*	i		31.08.2009
JB 24208/B161	*Ixobrychus minutus*	a	F	14.09.2009
BS 00283/B164	*Ixobrychus minutus*	i	M	31.05.2009

BS 00282/B165	*Jynx torquilla*	i	M	31.05.2009
FA 03764/B166	*Jynx torquilla*	i		30.08.2009
FA 06847/B167	*Jynx torquilla*	a		28.08.2009
FA 06841/B168	*Jynx torquilla*	a		25.08.2009
FA 06850/B169	*Jynx torquilla*	i		28.08.2009
FA 07910/B170	*Jynx torquilla*	i		13.09.2009
FA 06873/B171	*Jynx torquilla*	i		01.09.2009
FA 06826/B173	*Lanius collurio*	i		21.08.2009
FA 06824/B174	*Lanius collurio*	i		21.08.2009
FA 06751/B175	*Lanius collurio*	i		22.08.2009
FA 06799/B176	*Lanius collurio*	i		18.08.2009
FA 06781/B177	*Lanius collurio*	a	M	14.08.2009
FA 06848/B178	*Lanius collurio*	i		27.08.2009
FA 06852/B179	*Lanius collurio*	i		28.08.2009
FA 07909/B180	*Lanius collurio*	i		13.09.2009
FA 07905/B187	*Lanius collurio*	i		12.09.2009
FA 06892/B189	*Lanius collurio*	i		08.09.2009
DA 03757/B190	*Lanius minor*			22.08.2009
JB 23548/B191	*Locustella fluviatilis*	a		28.08.2009
JB 23374/B192	*Locustella luscinioides*	i		18.08.2009
JB 23371/B193	*Locustella luscinioides*	i		18.08.2009
JB 24183/B194	*Locustella luscinioides*	i		13.09.2009
JB 24045/B195	*Locustella luscinioides*	n		07.09.2009
JB 24024/B196	*Locustella luscinioides*	i		06.09.2009
JB 24189/B197	*Locustella luscinioides*	i		13.09.2009
JB 24024 /B198	*Locustella luscinioides*			14.09.2009
HA 15716/B199	*Luscinia luscinia*	a		21.08.2009
HA 15719/B200	*Luscinia luscinia*	i		23.08.2009
HA 15717/B201	*Luscinia luscinia*	i		22.08.2009
HA 15730/B202	*Luscinia luscinia*	i		31.08.2009
HA 15730/B203	*Luscinia luscinia*	i		09.09.2009
JB 23486/B205	*Luscinia svecica*	i	F	24.08.2009
YH 03032/B215	*Merops apiaster*	i		29.08.2009
YH 03030/B217	*Merops apiaster*	i		27.08.2009
YH 03048/B218	*Merops apiaster*	i		09.09.2009
YH 03038/B219	*Merops apiaster*	i		06.09.2009
YH 03051/B220	*Merops apiaster*	i		10.09.2009
YH 03039/B221	*Merops apiaster*	i		06.09.2009
YH 03035/B222	*Merops apiaster*	a		29.08.2009
YH 03036/B223	*Merops apiaster*	a	F	01.09.2009
YH 15747/B224	*Merops apiaster*	a		09.09.2009
YH 03040/B225	*Merops apiaster*	i		08.09.2009
YH 03044/B226	*Merops apiaster*	i		09.09.2009
YH 15746/B227	*Merops apiaster*	i		09.09.2009
YH 03052/B228	*Merops apiaster*	i		13.09.2009

YH 03049/B229	*Merops apiaster*	i		10.09.2009
FA 07907/B231	*Miliaria calandra*	n		13.09.2009
JB 24232/B232	*Motacilla flava*	i		16.09.2009
JB 24064/B233	*Motacilla flava*	a	M	07.09.2009
JB 24063/B234	*Motacilla flava*	a	M	07.09.2009
JB 24050/B235	*Motacilla flava*	i	M	07.09.2009
JB 24110/B236	*Motacilla flava*	a	F	09.09.2009
JB 24069/B237	*Motacilla flava*	a	M	07.09.2009
JB 24067/B238	*Motacilla flava*	a	M	01.09.2009
JB 24203/B239	*Motacilla flava*	i		13.09.2009
JB 24207/B240	*Motacilla flava*	i		14.09.2009
JB 24219/B241	*Motacilla flava*	i		14.09.2009
JB 24066/B242	*Motacilla flava*	i	M	08.09.2009
JB 24062/B243	*Motacilla flava*	i	M	07.09.2009
JB 24141/B244	*Motacilla flava*	i	M	10.09.2009
JB 24070/B245	*Motacilla flava*	i	M	07.09.2009
JB 24155/B246	*Motacilla flava*	a	M	11.09.2009
JB 24068/B247	*Motacilla flava*	a	F	07.09.2009
JB 24071/B248	*Motacilla flava*	i	M	07.09.2009
JB 23973/B249	*Muscicapa striata*	i		03.09.2009
JB 23181/B250	*Muscicapa striata*	n		13.09.2009
DA 03761/B252	*Oriolus oriolus*	i		01.09.2009
HA 15705/B254	*Passer domesticus*	i	M	16.08.2009
HA 15706/B255	*Passer domesticus*	i	M	16.08.2009
HA 15743/B257	*Passer domesticus*	i	M	17.09.2009
JB 24139/B258	*Parus major*	i	F	10.09.2009
JB 24034/B259	*Parus major*	a	M	06.09.2009
JB 24138/B260	*Parus major*	a	M	10.09.2009
JB 23303/B261	*Passer montanus*	n		14.08.2009
JB 23465/B262	*Passer montanus*			22.08.2009
JB 23456/B263	*Passer montanus*	n		21.08.2009
JB 23477/B264	*Passer montanus*	n		23.08.2009
JB 23466/B265	*Passer montanus*			22.08.2009
JB 24019/B266	*Passer montanus*	n		05.09.2009
JB 24230/B267	*Passer montanus*	n		15.09.2009
RA 26332/B268	*Phylloscopus collybita*	n		16.08.2009
RA 26774/B270	*Phylloscopus collybita*	i		27.10.2009
RA 26786/B271	*Phylloscopus collybita*	a		28.10.2009
RA 26779/B272	*Phylloscopus collybita*	n		28.10.2009
RA 26732/B273	*Phylloscopus collybita*	i		24.10.2009
RA 26785/B274	*Phylloscopus collybita*	i		28.10.2009
RA 26772/B275	*Phylloscopus collybita*	a		27.10.2009
RA 26776/B276	*Phylloscopus collybita*	a		27.10.2009
RA 26777/B277	*Phylloscopus collybita*	a		27.10.2009
RA 26775/B278	*Phylloscopus collybita*	a		27.10.2009

RA 26787/B279	*Phylloscopus collybita*	a		27.10.2009
RA 26760/B280	*Phylloscopus collybita*	i		26.10.2009
RA 26773/B283	*Phylloscopus collybita*	i		27.10.2009
RA 26357/B284	*Phylloscopus lorenzii*	n		06.09.2009
RA 26334/B285	*Phylloscopus trochilus*	i		23.08.2009
RA 26335/B286	*Phylloscopus trochilus*	n		23.08.2009
RA 26342/B287	*Phylloscopus trochilus*	a		27.08.2009
RA 26359/B290	*Phylloscopus trochilus*	a		11.09.2009
RA 26361/B291	*Phylloscopus trochilus*	i		14.09.2009
RA 26363/B292	*Phylloscopus trochilus*	a		15.09.2009
RA 26360/B293	*Phylloscopus trochilus*	i		13.09.2009
RA 26358/B294	*Phylloscopus trochilus*	a		11.09.2009
JB 23549/B295	*Phoenicurus phoenicurus*	n	F	28.08.2009
JB 23538/B296	*Phoenicurus phoenicurus*	i	M	27.08.2009
JB 24310/B297	*Phoenicurus phoenicurus*	i	M	17.09.2009
JB 24311/B298	*Phoenicurus phoenicurus*	i	F	17.09.2009
JB 23735/B299	*Phoenicurus phoenicurus*	i	M	01.09.2009
JB 24318/B300	*Phoenicurus phoenicurus*	i	F	17.09.2009
JB 24222/B301	*Phoenicurus phoenicurus*	a	M	15.09.2009
JB 24239/B302	*Phoenicurus phoenicurus*	i		15.09.2009
JB 24151/B303	*Phoenicurus phoenicurus*		F	11.09.2009
JB 24083/B304	*Phoenicurus phoenicurus*	i	M	08.09.2009
JB 24176/B306	*Phoenicurus phoenicurus*		F	13.09.2009
RA 26348/B307	*Phylloscopus sibilatrix*	i		30.08.2009
BS 00289/B308	*Pica pica*	i		30.10.2009
JB 25203/B309	*Prunella modularis*	i		25.10.2009
JB 23695/B310	*Oenanthe hispanica*	i	F	30.08.2009
JB 23560/B311	*Remiz pendulinus*	i		28.08.2009
JB 23580/B312	*Remiz pendulinus*	i		29.08.2009
JB 23561/B313	*Remiz pendulinus*	i		28.08.2009
JB 23559/B314	*Remiz pendulinus*	i		28.08.2009
JB 23492/B315	*Remiz pendulinus*	i		24.08.2009
JB 23730/B316	*Remiz pendulinus*	i		31.08.2009
JB 24221/B317	*Riparia riparia*	i		15.09.2009
JB 23995/B318	*Riparia riparia*	n		04.09.2009
JB 24204/B320	*Riparia riparia*	i		13.09.2009
JB 24205/B321	*Riparia riparia*	a		13.09.2009
JB 24218/B322	*Riparia riparia*	i		14.09.2009
JB 23537/B324	*Saxicola rubetra*	i	M	27.08.2009
JB 23487/B325	*Saxicola rubetra*	i	M	24.08.2009
JB 24236/B326	*Saxicola rubetra*	i		16.09.2009
JB 24119/B327	*Saxicola rubetra*	i	M	09.09.2009
JB 23722/B328	*Saxicola rubetra*	i	F	31.08.2009
JB 23725/B331	*Sylvia atricapilla*	a	F	31.08.2009
JB 24144/B333	*Sylvia atricapilla*	i	F	11.09.2009

JB 24152/B334	*Sylvia atricapilla*	i	F	11.09.2009
JB 23210/B336	*Sylvia borin*			31.05.2009
JB 23469/B337	*Sylvia borin*	a		23.08.2009
JB 23331/B338	*Sylvia borin*	a		16.08.2009
JB 23449/B339	*Sylvia borin*	a		21.08.2009
JB 23460/B340	*Sylvia borin*	i		22.08.2009
JB 23438/B341	*Sylvia borin*	i		21.08.2009
JB 23314/B342	*Sylvia borin*	i		15.08.2009
JB 23344/B345	*Sylvia borin*	i		17.08.2009
JB 23330/B347	*Sylvia borin*	a		16.08.2009
JB 23526/B349	*Sylvia borin*	a		26.08.2009
JB 23523/B353	*Sylvia borin*	i		26.08.2009
JB 24120/B355	*Sylvia borin*	i		09.09.2009
JB 24098/B366	*Sylvia borin*	i		08.09.2009
JB 24112/B367	*Sylvia borin*	i		09.09.2009
JB 24113/B370	*Sylvia borin*	i		09.09.2009
JB 23450/B372	*Sylvia communis*	i		21.08.2009
JB 23312/B374	*Sylvia communis*	i	M	15.08.2009
JB 23313/B375	*Sylvia communis*	i		15.08.2009
JB 23476/B377	*Sylvia communis*	i		23.08.2009
JB 23315/B379	*Sylvia communis*	i		15.08.2009
JB 23553/B380	*Sylvia communis*	i		28.08.2009
JB 23493/B381	*Sylvia communis*	i		24.08.2009
JB 23484/B382	*Sylvia communis*	a	M	24.08.2009
JB 23522/B383	*Sylvia communis*	i		26.08.2009
JB 23485/B385	*Sylvia communis*	i		24.08.2009
JB 23541/B386	*Sylvia communis*	i		27.08.2009
JB 24091/B388	*Sylvia communis*	i		08.09.2009
JB 24053/B389	*Sylvia communis*	i		07.09.2009
JB 23728/B391	*Sylvia communis*	i		31.08.2009
JB 23583/B392	*Sylvia communis*	a		28.08.2009
JB 23733/B393	*Sylvia communis*	i		31.08.2009
JB 24145/B394	*Sylvia communis*	i	M	11.09.2009
JB 23547/B395	*Sylvia curruca*	i		28.08.2009
JB 23515/B396	*Sylvia curruca*	a		25.08.2009
JB 23581/B397	*Sylvia curruca*	a		29.08.2009
JB 23509/B398	*Sylvia curruca*	n		25.08.2009
HA 15729/B400	*Sylvia curruca*	i		30.08.2009
JB 25206/B402	*Turdus merula*	i		26.10.2009

APPENDIX D: GEL IMAGES OF PCR REACTIONS AMPLIFIED WITH THE PRIMER PAIR 621-983

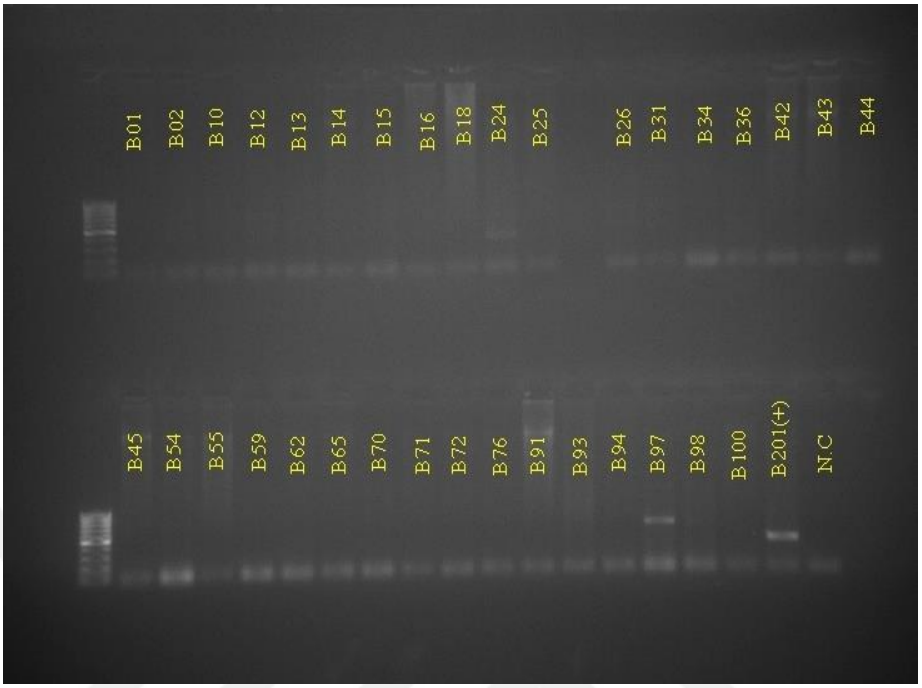


Figure D.1. Agarose gel of PCR products obtained with primer pair 621 – 983 #1

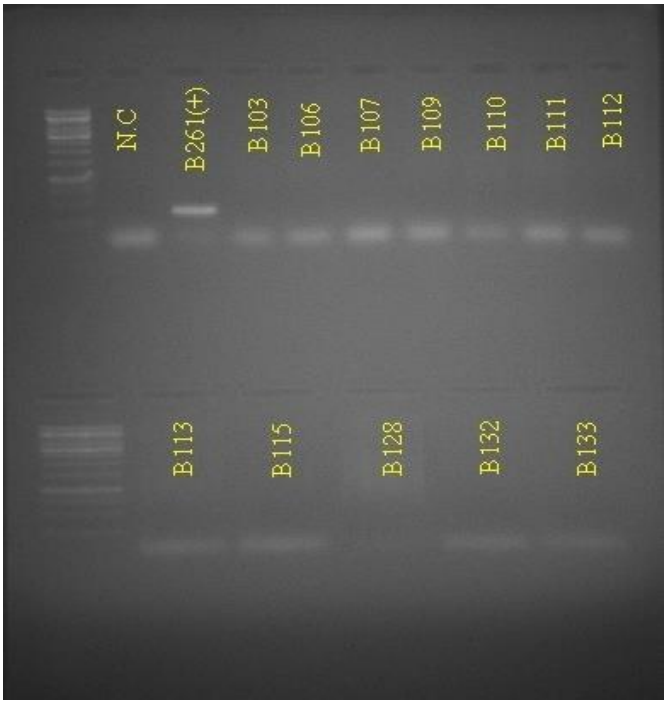


Figure D.2. Agarose gel of PCR products obtained with primer pair 621 – 983 #2

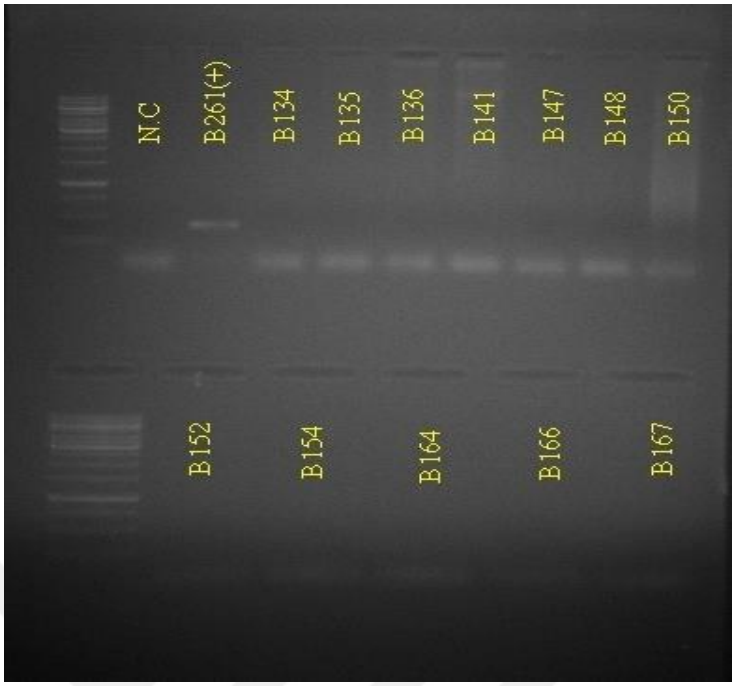


Figure D.3. Agarose gel of PCR products obtained with primer pair 621 – 983 #3

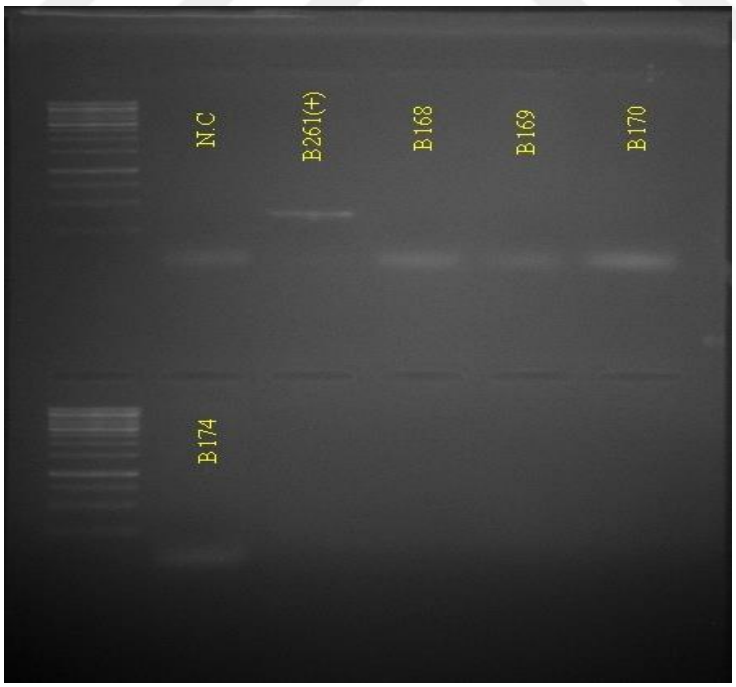


Figure D.4. Agarose gel of PCR products obtained with primer pair 621 – 983 #4

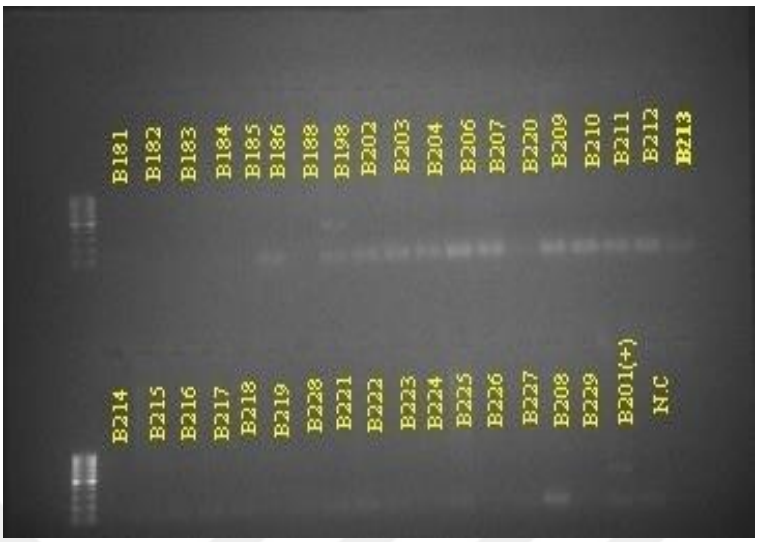


Figure D.5. Agarose gel of PCR products obtained with primer pair 621 – 983 #5

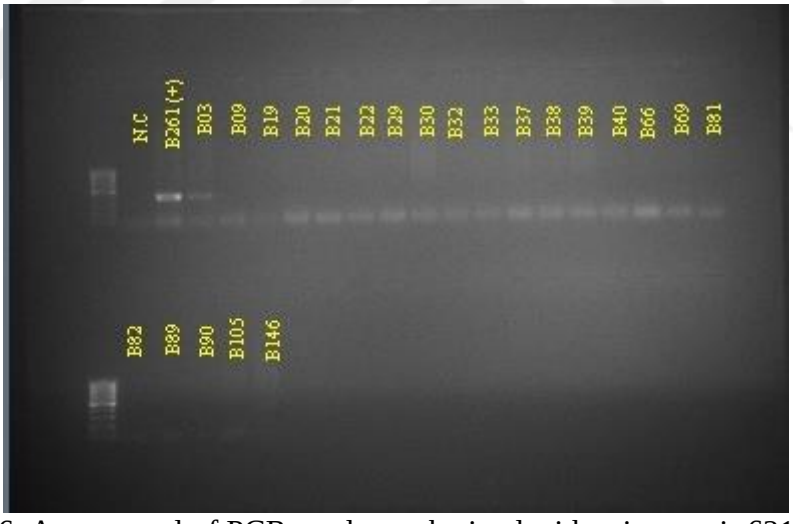


Figure D.6. Agarose gel of PCR products obtained with primer pair 621 – 983 #6

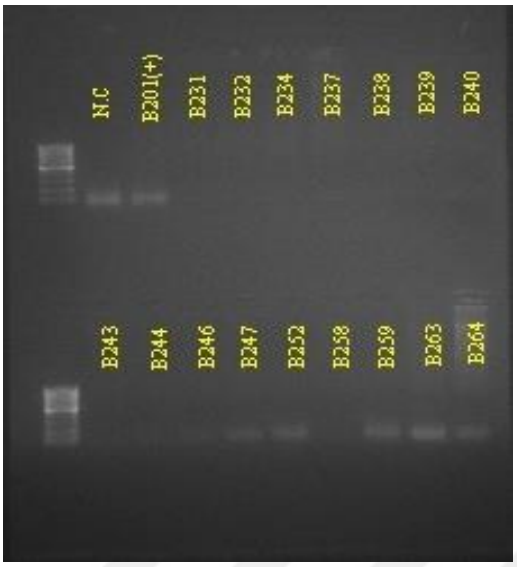


Figure D.7. Agarose gel of PCR products obtained with primer pair 621 – 983 #7

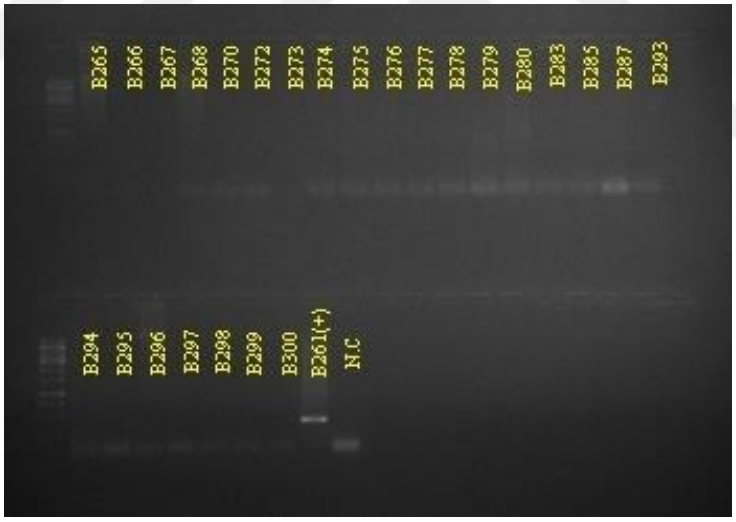


Figure D.8. Agarose gel of PCR products obtained with primer pair 621 – 983 #8

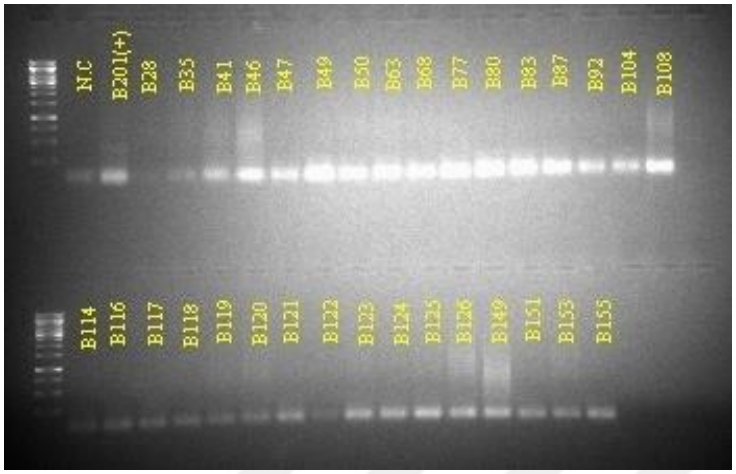


Figure D.9. Agarose gel of PCR products obtained with primer pair 621 – 983 #9

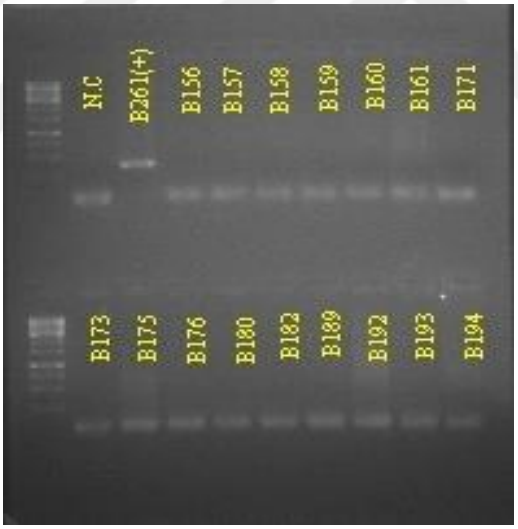


Figure D.10. Agarose gel of PCR products obtained with primer pair 621 – 983 #10

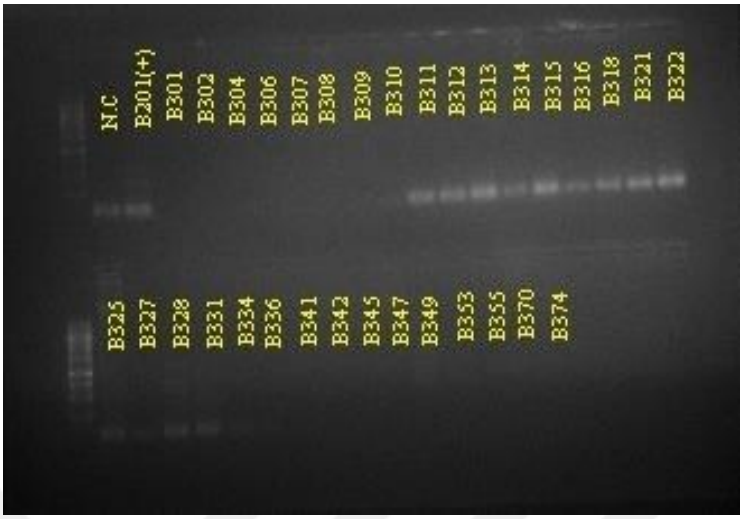


Figure D.11. Agarose gel of PCR products obtained with primer pair 621 – 983 #11

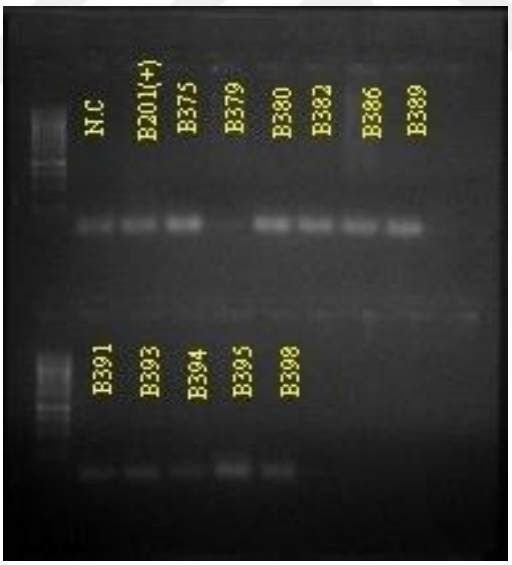


Figure D.12. Agarose gel of PCR products obtained with primer pair 621 – 983 #12

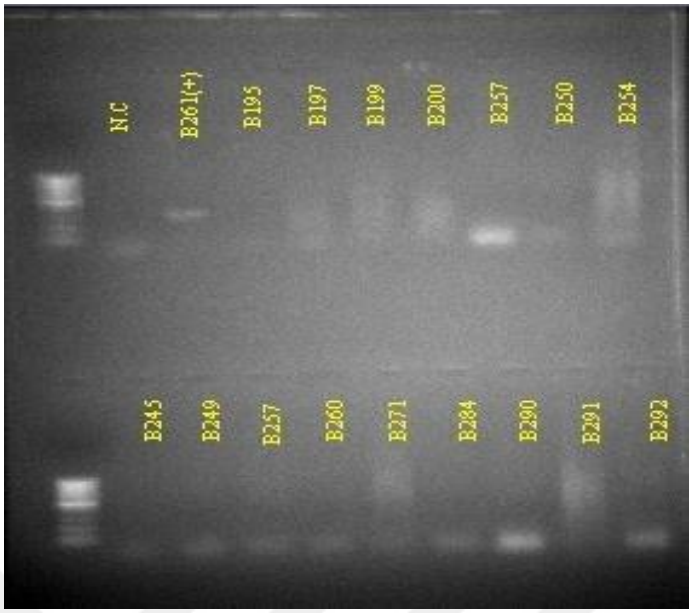


Figure D.13. Agarose gel of PCR products obtained with primer pair 621 – 983 #13

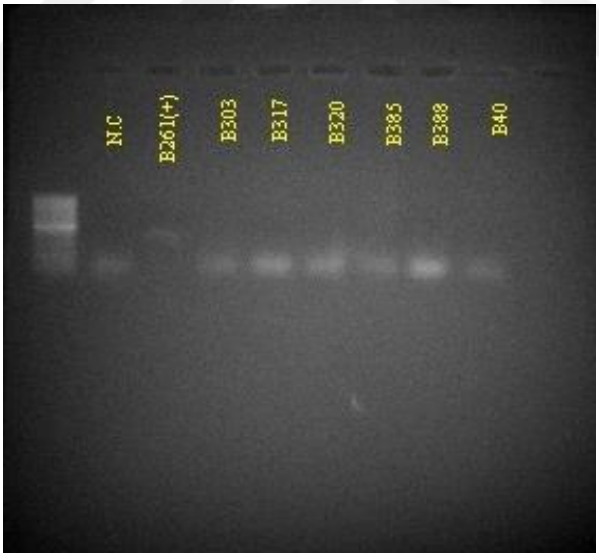


Figure D.14. Agarose gel of PCR products obtained with primer pair 621 – 983 #14

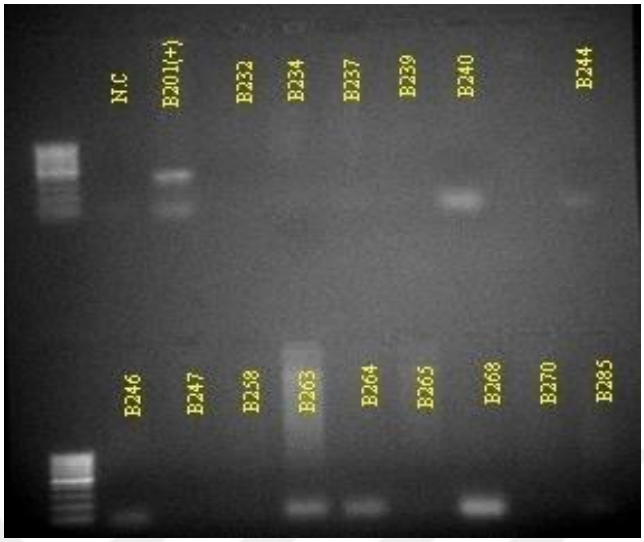


Figure D.15. Agarose gel of PCR products obtained with primer pair 621 – 983 #15

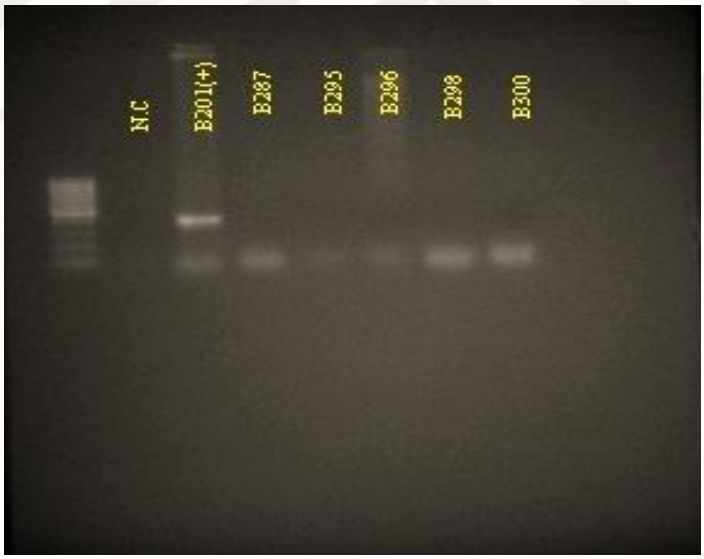


Figure D.16. Agarose gel of PCR products obtained with primer pair 621 – 983 #16

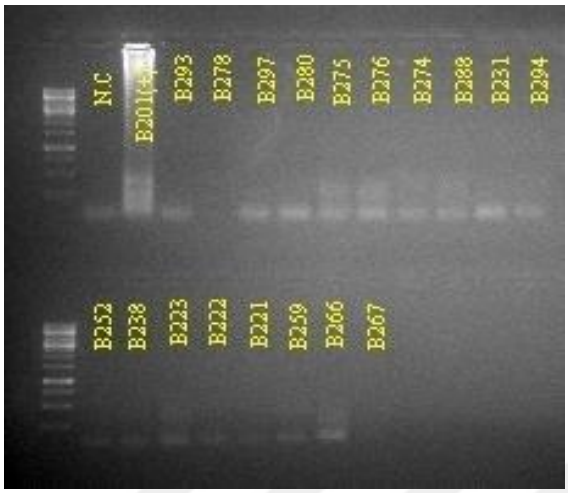


Figure D.17. Agarose gel of PCR products obtained with primer pair 621 – 983 #17